The global nutritional regulator CodY is an essential protein in the human pathogen Streptococcus pneumoniae

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6

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12

13   **Running title**:

14   Essentiality of CodY in *Streptococcus pneumoniae*

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18

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24   # Summary

25

26   CodY is a global regulator highly conserved in low-G+C Gram-positive bacteria. It plays a

27   key role in the adaptation of *Bacillus subtilis* to nutritional limitation through repression of a

28   large gene set during exponential growth and relief of repression upon starvation. In several

29   pathogenic bacteria, CodY regulates major virulence genes. Our interest in *Streptococcus*

30   *pneumoniae* CodY originates from our observations that the oligopeptide permease Ami was

31   involved in repression of competence for genetic transformation. We hypothesized that

32   peptide uptake through Ami feeds amino acid pools, which are sensed by CodY to repress

33   competence. As our initial attempts at inactivating *codY* failed, we launched an in-depth

34   analysis into the question of the essentiality of *codY*. We report that *codY* cannot be

35   inactivated unless a complementing ectopic copy is present. We obtained genetic evidence

36   that a recently published D39 *codY* knock-out contains additional mutations allowing survival

37   of *codY* mutant cells. Whole genome sequencing revealed mutations in *fatC*, which encodes a

38   ferric iron permease, and *amiC*. This combination of mutations was confirmed to allow

39   tolerance of *codY* inactivation. The *amiC* mutation is in itself sufficient to account for the

40   strong derepression of competence development observed in D39 *codY* cells.

Introduction

CodY is a global regulator highly conserved in low-G+C Gram-positive bacteria (Sonenshein, 2005). In *Bacillus subtilis*, the CodY regulon is large, encompassing nearly 200 genes (Molle *et al.*, 2003), most of which are repressed during exponential growth and induced when cells experience nutrient deprivation. In several pathogenic bacteria, CodY regulates major virulence genes (for review, see Sonenshein, 2005; Sonenshein, 2007). The repressor function of CodY (i.e. its DNA-binding activity) is activated by interaction with branched-chain amino acids (BCAAs), as originally shown *in vivo* in *Lactococcus lactis* (Guédon *et al.*, 2001). In species other than the Streptococci, Lactococci and Enterococci, CodY is also activated by interaction with GTP as first demonstrated (Ratnayake-Lecamwasam *et al.*, 2001) and further documented (Handke *et al.*, 2008) with the *B. subtilis* protein. In the latter bacterium, CodY is known to control not only metabolic pathways but also cellular processes, such as motility, sporulation, and competence for genetic transformation (Sonenshein, 2005; Sonenshein, 2007). In the latter case, CodY represses both *comK*, which encodes the master transcriptional activator of competence (*com*) genes, and *srfA*, a key operon for transcriptional activation of *comK* (Serror and Sonenshein, 1996).

Until recently, little was known regarding the role(s) of CodY in *Streptococcus pneumoniae*. Our interest in CodY stems from our previous observations that the oligopeptide permease Ami was, together with its dedicated oligopeptide-binding lipoproteins (Obl, i.e. AmiA, AliA, and AliB) (Alloing *et al.*, 1994), indirectly involved in the regulation of competence (Claverys *et al.*, 2000; Claverys and Håvarstein, 2002). Competence development of an *obl* mutant thus occurred at a ~50-fold reduced cell density compared to wild type (Alloing *et al.*, 1998). We hypothesized that the uptake of oligopeptides plays a key role in metabolic regulation in *S. pneumoniae*, by providing information on nutrient availability

66 (Claverys *et al.*, 2000). Exhaustion of nutrients would be sensed through a mechanism

67 involving peptide uptake by the Ami-Obl oligopeptide permease, followed by peptidase

68 digestion to release amino acids (aa) leading to replenishment of aa pools, which in turn affect

69 a global regulatory protein such as CodY (Claverys *et al.*, 2000). The above-mentioned

70 demonstration that BCAAs are effectors of CodY in several species would fit nicely with this

71 hypothesis since *S. pneumoniae* Obl proteins have different but overlapping specificities for

72 peptides including those containing BCAAs (Alloing *et al.*, 1994). Thus import of BCAAs (as

73 part of oligopeptides) by the Ami-Obl permease could directly impact CodY activity. This

74 situation would not be unprecedented since CodY-dependent repression of *comK* and *srfA* was

75 lost in an *opp* (oligopeptide permease) mutant of *B. subtilis* (Serror and Sonenshein, 1996). To

76 account for the negative impact of Ami-Obl on competence induction, we explicitly stated in

77 our model that CodY should repress competence (Claverys *et al.*, 2000).

78 To test this model, we initiated experiments aimed at inactivating *S. pneumoniae codY*.

79 Surprisingly, this gene turned out to be difficult to inactivate. However, *codY* inactivation had

80 recently been achieved through insertion of a trimethoprim resistance gene (*trim*) in strain

81 D39 (Hendriksen *et al.*, 2008). Microarray analysis using this mutant suggested that

82 pneumococcal CodY functions mainly as a transcriptional repressor, as 43 of the 47 genes

83 differentially expressed in the *codY*::*trim* mutant were found to be upregulated. The

84 inefficiency in constructing a *codY* knock-out in strain R6 prompted us to launch an in-depth

85 analysis of the question of the essentiality of *codY*. Here, we present our data establishing that

86 *codY* is essential in our laboratory strains, as well as in *S. pneumoniae* R6 and encapsulated

87 D39 strains. We provide genetic evidence that, in addition to the *codY* knock-out, the

88 *codY*::*trim* mutant used for transcriptome analysis of the CodY regulon and for virulence

89 studies (Hendriksen *et al.*, 2008) contains additional mutations (suppressors) allowing

90 survival of *codY* mutant cells. We document a strong derepression of competence in such

91   *codY*-mutant cells. Finally, we use comparative whole genome sequencing to identify

92   suppressor mutations in *fatC*, which encodes a ferric iron permease, and *amiC*. We confirm

93   that this combination of mutations allows tolerance of *codY* inactivation and discuss possible

94   reasons for CodY essentiality in *S. pneumoniae* in light of these observations.

95

96

97   Results

98

99   *Minitransposon insertion mutagenesis of* S. pneumoniae codY *is not possible*

100

101   A *codY* PCR fragment amplified with the primer pair MP188-MP189 (Table 1) was used as

102   target for *mariner* mutagenesis (Experimental procedures). Analysis of more than 50

103   minitransposon insertions through PCR revealed that only 6 clones produced an MP188-

104   MP189 fragment of which the size was consistent with that predicted for integration of the

105   *kan*[R] minitransposon (donated by plasmid pR410; Table 1). Localization of these 6 insertions

106   indicated that none of them had inserted into the *codY* gene (Fig. 1A). Given our previous

107   experience with *mariner* mutagenesis of the *ciaRH* (Martin *et al.*, 2000), *endA*, *comEAC*,

108   *comFAC*, *comGAB*, and *dprA* (Bergé *et al.*, 2002), as well as *cibABC* (Guiral *et al.*, 2005),

109   *comM-lytR* (Håvarstein *et al.*, 2006) and *radC* (Attaiech *et al.*, 2008) loci, we concluded that

110   the failure to isolate insertions in a locus occupying a central position on the targeted PCR

111   fragment and the biased distribution observed were strongly indicative of the essentiality of

112   *codY*, at least in the genetic background and under the plating conditions (CAT-agar) used. [It

113   is of note that a similar conclusion regarding the essentiality of *lytR* (*spr1759*) based on the

114   failure to isolate *mariner* insertions in this locus (Håvarstein *et al.*, 2006) was confirmed in a

115   further study (Johnsborg and Havarstein, 2009).]

116

117

118   *Construction of a strain harboring a second (ectopic) copy of* codY

119

120   The failure to inactivate *codY* through *mariner* mutagenesis prompted us to construct a strain

121   harboring a second copy of *codY* that should tolerate inactivation of one of the two copies.

122   The second copy was inserted at CEP (chromosomal expression platform) (Guiral *et al.*,

123   2006) under the control of the maltose-inducible promoter, P_M (Fig. 1B). The resulting strain

124   is referred to as *codY*$^{+/+}$ hereafter. The duplication of *codY* had no detectable effect on growth

125   and spontaneous competence induction when cells were grown with 1% maltose, i.e. under

126   conditions leading to full induction of the P_M promoter (Guiral *et al.*, 2006), suggesting that

127   the level of CodY attained with this induction system is not detrimental to the cell (data not

128   shown).

129

130

131   *Minitransposon insertions in* codY *are readily obtained in diploid (*codY$^{+/+}$*) cells*

132

133   A *codY*$^{+/+}$ strain (R2349) was then used as recipient for *mariner* mutagenesis of *codY* with

134   plasmid pR412 as donor of minitransposon (*spc*^{R} cassette; Table 1). In contrast to the failure

135   to inactivate *codY* using wildtype recipient cells, minitransposon insertions were readily

136   obtained in cells grown in maltose. A codY1-codY2 (Table 1) PCR fragment of which the

137   size was consistent with that predicted for integration of the *spc*^{R} minitransposon was

138   observed for 11 out of 20 randomly selected clones. Five of these insertions turned out to

139   inactivate *codY* as judged from their location (Fig. 1C) and from the inability to introduce

140   them at high frequency in a wildtype recipient (data not shown but see next section).

141   The isolation of minitransposon insertions in the genuine copy of *codY* suggested that the

142   ectopic copy placed at CEP was able to complement *codY* deficiency. This conclusion was

143   further strengthened by the observation that upon transformation of *codY*$^{+/+}$ cells with a PCR

144   fragment carrying the *codY*::*spc*^{3A} insertion (generated with the primer pair codYatg-

145   codYstop; Fig. 1C and Table 1), Spc^R transformants distributed about equally between the

146   genuine *codY* (e.g. strain R2424 the genotype of which is denoted *codY*$^{spc3/+}$; Fig. 2A) and the

147   ectopic CEP$_M$-*codY* loci (e.g. strain R2425 the genotype of which is denoted *codY*$^{+/spc3}$; Fig.

148   2B).

149

150

151   *Transformation frequencies of* codY$^{spc3/+}$ *in wildtype and* codY$^{+/+}$ *cells confirm* codY

152   *essentiality*

153

154   To unambiguously demonstrate the essentiality of *codY* in our strains, we then used

155   chromosomal DNA from a *codY*$^{spc3/+}$ strain (R2438) as donor in transformation of a wildtype

156   recipient. If *codY* is essential, the survival of transformants harboring the *codY*::*spc*^{3A} knock-

157   out mutation is predicted to rely on the simultaneous integration of CEP$_M$-*codY-kan* (i.e. a

158   double transformation event occurring independently as the two loci are not genetically

159   linked). On the other hand, a *codY*$^{+/+}$ recipient should readily accept the *codY* knock-out.

160   Transformation frequency should therefore be significantly reduced in wildtype cells

161   compared to a *codY*$^{+/+}$ recipient. In full agreement with this prediction, transformation of

162   *codY*::*spc*^{3A} occurred in *codY*$^{+/+}$ (strain R2350) cells with a frequency close to that of the *str41*

163   (Str^R) reference marker [Note that transformation of the *spc*^{R} cassette, which requires

164   integration of ~1.1 kb heterologous DNA, is expected to occur with a 2 to 3-fold reduced

165   frequency compared to the point-mutation reference marker *str41*] (Fig. 3A). In contrast, ~20-

166 fold reduction in the frequency of Spc^R transformants was observed with wildtype (R895)

167 cells (Fig. 3A). The observed frequency was close to that calculated as the product of

168 individual transformation frequencies of Spc^R and Kan^R. To verify that Spc^R transformants

169 obtained with the wildtype recipient had simultaneously acquired resistance to Kan, 10 Spc^R

170 transformants were isolated. All of them turned out to carry the CEP$_M$-*codY-kan* construct

171 (data not shown). Altogether, these data demonstrated that *codY* is an essential gene in *S.*

172 *pneumoniae* R800 and its derivatives.

173

174

175 codY *is also essential in R6 and in encapsulated D39*

176

177 R800 was originally derived from R6 through introduction of a suppressor mutation

178 (originating from strain Cl$_3$, another D39 derivative) (Tiraby *et al.*, 1975) that greatly

179 improved growth of *ami* mutants (Lefèvre *et al.*, 1979). This led us to wonder whether the

180 presence of this uncharacterized suppressor was responsible for the essentiality of *codY*. We

181 therefore introduced the CEP$_M$-*codY* construct in R6 (generating strain R2427) and measured

182 transformation frequencies of *codY*::*spc^{3A}* using *codY$^{spc3/+}$* chromosomal DNA as donor. Yield

183 of Spc^R transformants was significantly lower in R6 than in its *codY$^{+/+}$* derivative indicating

184 that *codY* is essential in this background as well (Fig. 3B).

185   Inactivation of *codY* was previously reported in the encapsulated *S. pneumoniae* D39 strain

186 (Hendriksen *et al.*, 2008). To check whether our negative data could be explained by the use

187 of unencapsulated strains, of different medium and/or plating conditions, we first tried to

188 transform D39 with an amiF1-kan1 PCR fragment carrying *codY*::*spc^{3A}* (Fig. 2B), using

189 previously described conditions for plating of *codY* $^-$ transformants on Columbia base agar

190 (Hendriksen *et al.*, 2008). No Spc^R transformants (i.e. <10^{-6}) could be obtained (data not

191   shown). We therefore adopted the same strategy as the one described above for R6.

192   Transformation of *codY*::*spc*[3A] using *codY*[spc3/+] chromosomal DNA as donor yielded ~20-fold

193   fewer Spc[R] transformants in D39 than in its *codY*^{+/+} derivative (strain TD73) indicating that

194   introduction of a *codY* knock-out in D39 also requires the simultaneous transfer of the

195   complementing ectopic CEP$_M$-*codY* gene (Fig. 3C). In full agreement with this interpretation,

196   Spc[R] transformants were also Kan[R]. We concluded that *codY* is also essential in encapsulated

197   D39 and that this gene must therefore be added to the list of essential pneumococcal genes

198   (Thanassi *et al.*, 2002).

199

200

201   *Re-investigation of the previously described* codY *knock-out reveals the presence of*

202   *additional suppressor mutations or a chromosomal rearrangement*

203

204   We then re-examined the previously constructed *codY*::*trim* mutant, strain D39 Δ*codY*

205   (Hendriksen *et al.*, 2008), as the transformation frequency upon mutant generation was very

206   low (W. Hendriksen, personal communication). PCR analysis of the *codY* chromosomal

207   region of strain D39Δ*cps*Δ*codY*, which was derived from D39Δ*codY* by transformation with a

208   PCR fragment harboring the Δ*cps*::*kan* cassette, as previously described (Bootsma *et al.*,

209   2007), confirmed the published structure (data not shown). Preliminary attempts using

210   D39Δ*cps*Δ*codY* chromosomal DNA as donor revealed that transformation of the *codY*::*trim*

211   construct into R800 derivatives occurred only at very low frequency (data not shown). To

212   facilitate the comparison with transformation experiments reported above, we replaced the

213   *codY*::*trim* construct with the *codY*::*spc*[3A] insertion (using as donor a codY1-codY2 PCR

214   fragment) and we introduced the *str41* reference marker (using as donor a PCR fragment

215   amplified with the rpsL_3-rpsL_4 primer pair; Table 1) thus generating strain TD81. Then,

216   using TD81 chromosomal DNA as donor, we compared transformation frequencies of

217   *codY*::*spc*^{3A} in the same pairs of *codY*$^+$ and *codY*$^{+/+}$ derivatives of R800, R6 and D39 used in

218   Fig. 3. Transformation frequencies in *codY*$^+$ strains were reduced by 544, 762, and 351 fold,

219   respectively in R800, R6, and D39, compared to their *codY*$^{+/+}$ isogenic derivatives (Fig. 4A-

220   C). These very large reductions in transformation frequency (compared to the ~20-fold

221   reduction observed when the simultaneous transfer of *codY*::*spc*^{3A} and CEP$_M$-*codY* from a

222   *codY*$^{spc3/+}$ donor was required; Fig. 3) indicated that transfer of the *codY*::*spc*^{3A} from strain

223   TD81 must be accompanied by the transfer of more than one point mutation. Alternatively,

224   the very low transformation frequency could be accounted for by the requirement for a

225   simultaneous chromosomal rearrangement. We concluded that the original D39Δ*cps*Δ*codY*

226   strain either contained two additional suppressor mutations that are presumably acting

227   together to compensate for the absence of CodY and restore cell viability, or harbored a

228   chromosomal rearrangement allowing survival in the absence of CodY. We tentatively named

229   it/them *socY* (for <u>s</u>uppressor <u>o</u>f <u>codY</u>).

230   To get a possible insight into the molecular nature of the mutation(s) involved, we carried

231   the same transformation experiments but with a pair of *hex* mutant derivatives of R800 (strain

232   R1818 and its *codY*$^{+/+}$ derivative, R2428). The Hex system of *S. pneumoniae* is known to

233   correct out some mismatches at the donor-recipient heteroduplex stage in transformation

234   (Claverys and Lacks, 1986). It is particularly efficient at correcting transition mismatches (i.e.

235   A/C or G/T) as well as short frameshifts (Gasc *et al.*, 1989). Transformation frequencies with

236   *hex*$^-$ recipient cells were improved ~4.5 fold (125 versus 544 fold reduction in the *codY*$^+$

237   parent compared to the *codY*$^{+/+}$ derivative, in *hex*$^-$ and *hex*$^+$ strains respectively; Fig. 4D),

238   which suggests that one of the *socY* mutations is recognized by the Hex system and is

239   therefore possibly a transition or a short frameshift. Alternatively, the recombination event

240   leading to the putative chromosomal rearrangement may involve the formation of a

241   heteroduplex intermediate harboring mismatches susceptible to the Hex system.

242

243

244   *Effect of* codY *inactivation on spontaneous competence development*

245

246   To characterize the role of *S. pneumoniae* CodY with respect to growth and the regulation of

247   competence, we first tried to deplete CodY making use of a *codY*$^{spc/+}$ strain, i.e. a strain in

248   which the only functional copy of *codY* was under the control of the P_M promoter. In light of

249   the failure to obtain a functionally significant depletion of CodY (Supplementary results), we

250   chose to characterize a *codY socY* strain with respect to spontaneous competence development

251   and growth. Competence was monitored throughout incubation at 37°C by using a

252   transcriptional fusion of the *luc* gene, which encodes luciferase, to the *ssbB* gene. The latter is

253   known to be specifically induced at competence. The *ssbB*::*luc* fusion thus reports on

254   competence through light emission by luciferase (Prudhomme and Claverys, 2007). We

255   introduced the *ssbB*::*luc* transcriptional fusion (using R895 chromosomal DNA as donor) in

256   strains D39Δ*cps* and D39Δ*cps*Δ*codY socY* thus generating strains TD82 and TD83,

257   respectively (Table 1). We then compared competence profiles of strains TD82 and TD83

258   during growth in C+Y medium with initial pH values between 6.48 and 7.26, since

259   spontaneous competence induction is known to be strongly dependent on the initial pH. For

260   instance, initial pH values between 6.8 and 8.0 affected the timing of occurrence and the level

261   of competence (Chen and Morrison, 1987). While the wildtype parent developed spontaneous

262   competence only in cultures with initial pH values above 7.0 (Fig. 5A-B), the *codY* mutant

263   could develop competence under acidic conditions, down to an initial pH value of 6.70 (Fig.

264   5C-D). It is of note that despite the presence of the *socY* suppressor, *codY* mutant cells grew

265   more slowly than wildtype cells in C+Y medium (Fig. 5A-D). *codY* mutant cells thus entered

266   the stationary phase of growth after ~270 min incubation compared to ~170 min for wildtype

267   cells. This >50% increase in generation time might indicate that CodY plays an important role

268   in the regulation of pneumococcal growth. Despite the fact that *codY* mutant cells grew more

269   slowly than wild type, they developed competence at about the same time (e.g. maximum

270   competence after 87 versus 82 min incubation at pH 7.26; Fig. 5A and 5C), which

271   corresponded to OD_{492} values of 0.083 and 0.115, respectively. This observation was also

272   consistent with upregulation of competence in the *codY* mutant. The strong competence-

273   upregulated (*cup*) phenotype (Martin *et al.*, 2000) displayed by *codY* mutant cells would be

274   consistent with the hypothesis that CodY normally represses competence under acidic

275   conditions in wildtype cells. However, the uncharacterized suppressor mutations (*socY*) in

276   strain TD83 could also be responsible for the observed *cup* phenotype. This prompted us to

277   investigate the phenotype of *socY* (*codY*$^+$) cells.

278

279

280   *Impact of* socY *on spontaneous competence development*

281

282   First, to establish whether restoration of *codY*$^+$ would be tolerated in a *socY* genetic

283   background, strain TD83 was transformed with R2644 chromosomal DNA. R2644 carries the

284   *spc*^{90C} insertion immediately upstream of the *codY*$^+$ gene (see Experimental procedures).

285   Integration of the *spc*^{R} cassette by transformation could thus be accompanied by the removal

286   of the adjacent *codY*::*trim* cassette. A failure to survive of Trim^S (i.e. *codY*$^+$) excisants should

287   result in a drastic reduction in the number of Spc^R transformants, since recombination events

288   leading to *spc* integration without the simultaneous deletion of *trim* are rare due to the reduced

289   distance between the two cassettes (105 bp). The high Spc^R to Str^R transformant ratio

290   observed (average value 0.32±0.06) was consistent with the simultaneous occurrence of both

291   integration (of the *spc* cassette) and excision (of the *trim* cassette) events, and suggested that

292   *socY* (*codY*⁺) cells are viable. To establish this, 4 of 4 randomly chosen Spc^R transformants

293   were first checked to be Trim^S. Then, they were shown to readily re-accept *codY::trim* when

294   transformed with TD80 chromosomal DNA (Trim^R/Str^R average ratio of 0.25±0.08 over 15

295   independent cultures). These data confirmed that the Spc^R Trim^S transformants were still *socY*

296   and indicated that none of the two suppressor mutations or the putative chromosomal

297   rearrangement is detrimental to pneumococcal cells when CodY is present. One of the Spc^R

298   Trim^S clones was retained and named strain TD95. The *spc*^{90C} cassette was then removed

299   from strain TD95 by transformation with a PCR fragment generated on R800 chromosomal

300   DNA with the MP188-MP189 primer pair, followed by phenotypic expression and

301   segregation in liquid culture (C+Y medium) for 4.5 hours, and plating on CAT-agar without

302   antibiotic. Individual colony screening was carried out to isolate a Spc^S clone, TD96. Upon

303   transformation of TD96 with TD80 chromosomal DNA, a Trim^R/Str^R ratio of 0.42±0.07 was

304   observed indicating that TD96 had remained *socY*.

305   The competence profile of TD96 during growth in C+Y medium with initial pH values

306   between 6.48 and 7.26 was compared with that of TD82 (wild type) and TD83 (*codY::trim*

307   *socY*). TD96 *socY* (*codY*⁺) cells grew more rapidly than parental *codY::trim socY* cells, but

308   still more slowly than wild type (entry into stationary phase after ~210 min versus ~170 min;

309   Fig. 5E-F). Interestingly, *socY* cells displayed a stronger *cup* phenotype than *codY socY* cells

310   as judged first from their ability to develop spontaneous competence at pH 6.60 (Fig. 5F) and

311   second, from the very early development of competence (36 min at pH 7.26) compared to

312   both wildtype and *codY socY* cells (Fig. 5). Thus, the *socY* mutations alone confer a strong

313   *cup* phenotype. The net effect of *codY* inactivation in this background is to attenuate this *cup*

314   phenotype. From these data, it is therefore difficult to conclude that CodY acts as a repressor

315   of competence in pneumococcal cells.

316

317

318   *Whole genome sequence comparison suggests* fatC *and* amiC *mutations suppress inviability*

319   *of* codY *mutants*

320

321   In an attempt to identify the *socY* (suppressor) mutations, whole genome resequencing of

322   strains D39 and D39Δ*codY* was performed (Experimental procedures). Compared to the

323   previously published D39 NCTC 7466 genome (Lanie *et al.*, 2007), the D39Δ*codY* strain had

324   14 mutations also found in its parent. As our D39 was obtained originally from NCTC (via T.

325   Mitchell), these mutations presumably have arisen during laboratory cultivation. None of

326   them appeared to alter an important function (Table S1), which is consistent with the fact that

327   this D39 strain displayed full virulence in a mouse model (Hendriksen *et al.*, 2008).

328   The *codY* mutant had also mutations flanking the *trim* cassette [a C→T transition, a

329   GT→AGC frameshift and a T→A transversion, respectively at positions -175, +681/682 and

330   +724 (positions are given with respect to the ATG of *codY*)]. All these changes occurred in

331   between the *trim* cassette and the oligonucleotide primers used to amplify the *codY* region

332   (Hendriksen *et al.*, 2008), and were presumably introduced during polymerase chain reaction.

333   The transition mutation is in itself sufficient to lower the frequency of integration of the

334   *codY*::*trim* cassette by ~5-fold during transformation of mismatch-repair proficient strains.

335   Most relevant with respect to the question of CodY essentiality, the *codY* mutant had, in

336   addition, a mutation in *fatC* and a second, variable mutation in *amiC* (Fig. 6A). The former

337   mutation (a C→T transition), *fatC*^{C496T} changed a CAA (Gln) codon into TAA (stop) in a gene

338   belonging to the *fatD-fatC-fecE-fatB* operon (*spd_1649-1652* in D39; *spr1684-1687* in R6;

339   *sp1869-1872* in TIGR4) (Fig. 6A, top). This operon [also called *piuBCDA* or *pit1* (Brown *et*

340   *al.*, 2002)] encodes the major ferric iron/heme [*fatB* has been shown to bind hemin (Tai *et al.*,

341   2003)] transporter of *S. pneumoniae* (Ulijasz *et al.*, 2004). The $fatC^{C496T}$ change is predicted to

342   result in the synthesis of a truncated FatC protein (165 instead of 318 aa) and was detected in

343   100% of forward and reverse reads. As concerns the base variations in *amiC*, which encodes a

344   598-aa protein and belongs to the *amiACDEF* operon (*spd_1671-1667* in D39; *spr1707-1703*

345   in R6; *sp1891-1887* in TIGR4), three types of mutations were observed: two single-base

346   changes (#2 and #3 in Fig. 6A, bottom) and a more complex mutation (#1 in Fig. 6A, bottom).

347   These variations were detected in only a fraction of sequence runs. Mutations #2 ($amiC^{G1438T}$)

348   and #3 ($amiC^{G1459T}$) change a GAC (Asp) codon into TAC (Tyr) (aa 480 and 487,

349   respectively); these mutations were detected in 30.8% of forward and 11.1% of reverse reads

350   (total with variation: 19%), and in 57.1% of forward and 40.9% of reverse reads (total with

351   variation: 47%), respectively. Interestingly, both changes affect an Asp aa conserved in the

352   entire family of Opp (oligopeptide), Dpp (dipeptide) and App (nickel) permeases (our

353   observations), which suggests that they are functionally important and that the corresponding

354   AmiC proteins may have lost their activity. The complex mutation #1 consisted of a G→TT

355   frameshift ($amiC^{G246TT}$) and the almost adjacent A→C change ($amiC^{A248C}$); both mutations

356   shared the same sequencing parameters (detection in 25.0% of forward and 33.3% of reverse

357   reads), which strongly suggests that they resulted from a single mutational event (total with

358   variation, 29%). This mutation could thus be described as a GGA→TTGC change. [In fact,

359   this change had already been observed during analysis of mismatch repair specificity in *S.*

360   *pneumoniae* and referred to as *amiA29* (at this time, the *ami* locus was thought to be a single

361   gene, hence the name *amiA*) (Gasc *et al.*, 1989) (for a molecular explanation of this

362   mutational event, see Fig. S2).] This complex mutation is predicted to result in the synthesis

15

363   of a truncated protein harboring the first 81 aa of AmiC fused to 13 'new' aa (resulting from

364   the frameshift).

365   At first sight, the presence of changes #1, #2 and #3 in only a fraction of sequence runs

366   appeared puzzling and suggested their late occurrence in an original $\Delta codY\ fatC^{C496T}$ clone.

367   As a first attempt to establish whether an *ami* mutation was required for tolerance of *codY*, we

368   analyzed the *ami* locus of D39$\Delta cps\Delta codY$, considering it a subclone of the original D39$\Delta codY$

369   strain since it was generated by transformation of this strain with a PCR fragment harboring

370   the Δcps::*kan* cassette. Taking advantage of the fact that *amiC-F* mutations normally confer

371   resistance to methotrexate (Mtx^R), transformation of an Mtx^S strain (R1501) with a series of

372   PCR fragments covering the *ami* region of strain D39$\Delta cps\Delta codY$ was used to localize any *ami*

373   mutation (Fig. S3). Transformation data were consistent with the presence of mutation #2 or

374   #3 in strain D39$\Delta cps\Delta codY$; DNA sequencing confirmed that this strain had inherited the

375   latter mutation ($amiC^{G1459T}$). These data prompted us to directly confirm the mixed structure

376   population with respect to *amiC* of the original D39$\Delta codY$ strain. An aliquot of the original

377   stock was plated, 16 individual clones were picked and *amiC* (and *fatC*) regions were

378   sequenced (Table S2). All clones turned out to harbor one of the three *amiC* mutations

379   identified during whole genome sequencing (and 11 out of 11 clones sequenced carried the

380   *fatC* mutation). Interestingly, the overall distribution between the three types was in very good

381   agreement with that inferred from the analysis of genome sequence data (Table S2). It is also

382   of note that 3 clones harbored change #1 (i.e. both $amiC^{G246TT}$ and $amiC^{A248}$), which provided

383   support to the view that a single molecular event accounts for a complex change (Fig. S2).

384   Altogether, these results strongly suggested that inactivation of *amiC* was required for

385   tolerance of *codY* inactivation. We tentatively concluded from these observations that the

386   combination of *fatC* and *amiC* mutations suppresses inviability of *codY* mutants.

387

388

The fatC amiC *combination allows tolerance of* codY *inactivation*

390

To confirm this conclusion, we generated *mariner* insertion mutants in *fatC* (Fig. S4). Then,

taking advantage of the availability of the well-characterized *amiC9* mutation ($amiC^{C355T}$)

[previously named *amiA9* (Gasc *et al.*, 1989)] which changes a CAA codon (Gln) into a TAA

(stop) codon leading to the synthesis of a truncated AmiC protein (118 aa), we investigated

the ability of recipient cells harboring the *fatC*::*cat*^{23C} cassette and/or the *amiC9* mutation

(Fig. 6A) to accept *codY* inactivation.

    Strain (R246) and its *amiC9* (R3003), *fatC*::*cat*^{23C} (R3002) and *amiC9 fatC*::*cat*^{23C}

(R3004) derivatives were used as recipients for the *codY*::*trim* or *codY*::*spc* cassettes,

respectively carried on TD80 and TD81 chromosomal DNA. These recipients contained a

hexA$^-$ mutation, which impairs mismatch repair, firstly to ensure similar integration frequency

of the cassettes since transfer of the *trim* cassette is otherwise predicted to be reduced by the

Hex system due to the presence of flanking mismatches (see above); secondly to equalize

transformation frequencies of *amiC*^{G1459T} and *fatC*^{C496T} point mutations.

    The *amiC fatC* double mutant derivative readily accepted the *codY* (*spc* or *trim*) cassette

with the expected frequency relative to the reference marker *str41* (~0.20), while introduction

of the cassette into its wildtype parent occurred with a ~45-fold reduced frequency (Fig. 6B).

As expected, *amiC* and *fatC* single mutants displayed a ~5 to 10-fold reduction in

transformation frequency of the *codY* cassette compared to *amiC fatC* cells. The latter

reduction reflected the need for co-transformation of the *fatC*::*cat*^{23C} and *amiC9* mutation,

respectively, while co-transformation of both *amiC* and *fatC* together with the *codY* cassette

was required in wildtype cells. Altogether, these results demonstrated that the *amiC fatC*

combination fully suppressed the inviability of *codY* mutant cells.

413   Growth and spontaneous competence development of wild type (TD82) and *amiC9*

414   (TD84), *fatC*::*cat^23C* (TD138), *amiC9 fatC*::*cat^23C* (TD135) and *amiC9 fatC*::*cat^23C codY*::*trim*

415   (TD154) mutant strains were then compared (Fig. S5). Both *fatC* and *amiC* mutant strains

416   displayed a *cup* phenotype, consistent in the latter case with the phenotype of *obl* mutants

417   (Alloing *et al.*, 1998). The *cup* phenotype of the double mutant was most similar to that

418   observed with the *amiC* mutant (Fig. S5) and with the *socY* strain (Fig. 5E-F). Finally, while

419   *codY* inactivation resulted in slower growth confirming the observation in Fig. 5, it did not

420   significantly attenuate the *cup* phenotype observed with the *amiC fatC* double mutant

421   (compare TD135 and TD154, Fig. S5).

422

423

424   Discussion

425

426   CodY is a pleiotropic regulator in low-GC Gram-positive bacteria involved in the control of

427   different processes like aa uptake, competence, sporulation and virulence. We first established

428   that *codY* is an essential gene in the D39 strain of *S. pneumoniae* and in several of its

429   laboratory derivatives by using different genetic approaches (*mariner* mutagenesis,

430   complementation, transfer of mutations in different genetic backgrounds; Fig. 1-3). We then

431   showed that a previously obtained *codY* mutant (D39Δ*codY*) used to identify the CodY

432   regulon (Hendriksen *et al.*, 2008) contains additional suppressor mutations, called *socY*, one

433   of which was concluded to correspond to a transition mismatch or a frameshift (Fig. 4). We

434   also showed that both the *codY socY* and *socY* strains displayed a competence upregulated or

435   *cup* phenotype (Fig. 5).

436   Whole genome sequencing of strains D39 and D39Δ*codY* undertaken to identify the *socY*

437   suppressors revealed the presence of mutations in *fatC* and *amiC*, which encode the

438   membrane permease component of ferric iron/heme and oligopeptide ABC transporters,

439   respectively (Fig. 6A). This combination of mutations was unambiguously demonstrated to be

440   required to allow tolerance of *codY* inactivation (Fig. 6B). These results fully confirm the

441   conclusions from *codY* transfer experiments, including the presence of a mutation susceptible

442   to mismatch repair (Fig. 4D), i.e. the *fatC*^{C496T} transition.

443

444

445   *Proposed scenario for the initial stabilization/survival of D39Δ*codY *cells*

446

447   Genome sequence clearly indicated that the *fatC* mutation arose first. However, genome

448   sequence data also strongly suggested that inactivation of *amiC* is absolutely required for

449   tolerance of *codY* inactivation. The finding that three different *amiC* mutations occurred in the

450   D39Δ*codY* strain, as confirmed by individual subclone analysis (Table S2), implied a strong

451   selection pressure (growth advantage) for *amiC* mutant derivatives. The *amiC* mutations

452   presumably arose independently in an otherwise *codY fatC* lineage.

453   It is of note that the D39Δ*codY* strain described in Hendriksen *et al.* (2008) was obtained

454   by backcrossing D39 using chromosomal DNA isolated from an initial D39Δ*codY*::*trim*

455   transformant. Despite this careful strategy, D39Δ*codY*::*trim* turned out to contain two

456   additional mutations, as shown in this study. While a second round of transformation

457   normally eliminates unlinked mutations, it is obviously unable do so when the unlinked

458   mutations are absolutely required for survival. In addition, we noticed that the two suppressor

459   mutations are not very distant. The two closest mutations (*fatC*^{C496T} and *amiC*^{G1459T}), located

460   17,987 nt apart, could frequently be carried by the same DNA fragment. Careful comparison

461   of transformation frequencies with a well-characterized reference marker (preferably present

462   on the same chromosomal DNA) is thus the only way to establish unambiguously that any

463   construct is well tolerated and does not affect cell viability.

464

465

466   *CodY,* socY *and competence*

467

468   It was implicit in our working hypothesis connecting competence regulation to oligopeptide

469   uptake via a global regulator (like CodY) sensing aa pools (see Introduction) that this

470   regulator would act as a repressor of competence (Claverys *et al.*, 2000). Our data rather

471   suggest that, in contrast to this expectation, inactivation of *codY* has no major effect on

472   spontaneous competence, most of the effects seen being attributable to the *socY* mutations

473   (Fig. 5 and Fig. S5). In fact, the *amiC* mutation is in itself sufficient to account for the strong

474   derepression of competence development observed in D39Δ*codY* cells (Fig. S5). It is of note

475   that despite the *cup* phenotype documented in this study for the D39Δ*cps*Δ*codY* strain (Fig. 5),

476   previous transcriptome analysis did not reveal induction of any *com* gene (Hendriksen *et al.*,

477   2008); presumably, culture conditions used for mRNA extraction did not lead to differential

478   induction of the *com* regulon between the *codY* mutant and its parent, competence being either

479   similarly induced or repressed for both strains. While normal Ami functioning somehow leads

480   to competence repression, CodY is either neutral (Fig. S5) or possibly acts as an activator of

481   competence, the latter conclusion being suggested by the attenuation of the *cup* phenotype of

482   *amiC fatC* cells upon introduction of the *codY* knock-out (Fig. 5). If confirmed, the role of *S.*

483   *pneumoniae* CodY with respect to competence regulation would thus differ from that of its *B.*

484   *subtilis* orthologue, which represses competence (Serror and Sonenshein, 1996), suggesting

485   that the impact of nutrient deprivation on competence induction is opposite in *B. subtilis* and

486   *S. pneumoniae* or at least that nutritional signals are conveyed in a very different way in these

487   two species. However, since the impact of *codY* inactivation on competence could so far be

488   evaluated only in complex genetic backgrounds (i.e. mutant for both *amiC* and *fatC*), further

489   work using different approaches, such as a transient depletion of CodY, would be necessary to

490   evaluate the exact role of CodY in the regulation of pneumococcal competence.

491

492

493   *CodY,* socY *and previous transcriptome studies*

494

495   The finding that the D39Δ*codY* strain used in a previous study (Hendriksen *et al.*, 2008) was

496   in fact a *codY amiC fatC* triple mutant raises the question of the respective contribution of

497   each mutation to the phenotypes previously attributed solely to the inactivation of *codY*. As

498   concerns transcriptome data, upregulation of *aliA* (*aliB* was also upregulated but only 1.8-

499   fold), *amiA-amiC-amiD* and *fatD-fatC-fecE-fatB* was reported in Δ*codY* (Hendriksen *et al.*,

500   2008). Binding of CodY to P_{amiA} (as well as to P_{aliB}) was consistent with a direct regulation of

501   oligopeptide uptake by CodY. CodY thus exhibited a strong affinity for P_{amiA} although the

502   effect of BCAA addition was limited (1.56-fold versus 2 to 20-fold enhancement for other

503   promoters). On the other hand, the *Kd*s (the CodY concentration at which 50% of the

504   fragment is shifted) for the P_{fatD} promoter was higher than the highest concentration tested

505   (2,000 nM) indicative of a rather weak affinity and was unaffected by addition of BCAAs,

506   contrary to most other CodY-regulated promoters (Hendriksen *et al.*, 2008). Possibly, the

507   binding of CodY to P_{fatD} is affected in the presence of other regulators (see below), as *fat*

508   regulation is clearly multifactorial (Ulijasz *et al.*, 2009). Alternatively, since Δ*codY* cells used

509   to prepare mRNA for transcriptome analysis were also mutant for *fatC* (i.e. they lacked the

510   main iron transporter of *S. pneumoniae*), the upregulation of the *fatD-fatC-fecE-fatB* operon

511   could be a reflection of (inefficient) attempts at iron homeostasis by increasing expression of

21

512   this transporter rather than the consequence of a lack of repression in the absence of CodY.

513   The same reasoning may apply to *dpr* gene expression. This gene (*spd_1402* in D39; *spr1430*

514   in R6; *sp1572* in TIGR4) encodes a conserved iron storage-peroxide resistance protein

515   (Ulijasz *et al.*, 2004) that is possibly essential (Pericone *et al.*, 2003). It was found to be

516   downregulated in the *codY* mutant (Hendriksen *et al.*, 2008). Depletion of iron in *codY* mutant

517   cells resulting from *fatC* inactivation could possibly account for the observed reduction in *dpr*

518   expression without implying any direct regulation by CodY. This would be consistent with the

519   failure to identify a sequence resembling the CodY box upstream of the *dpr* gene. Clearly,

520   additional work is necessary to establish whether CodY regulates *dpr* expression directly or

521   indirectly.

522

523

524   *CodY,* socY *and previous virulence studies*

525

526   Similarly, the presence of *amiC* and *fatC* mutations in the D39Δ*codY* strain used in a previous

527   study (Hendriksen *et al.*, 2008) raises the question of their respective contribution to the

528   virulence phenotypes previously attributed solely to *codY* inactivation. The importance of iron

529   for bacterial growth and virulence is well established. Thus, signature-tagged mutagenesis

530   (STM) screens for genes essential during pneumococcal pneumonia identified insertions in

531   genes belonging to each of the three iron transporters characterized in *S. pneumoniae* (Brown

532   *et al.*, 2001; Brown *et al.*, 2002), *piuB* (i.e. *fatD*), *pitB* (*pitADBC* operon) and *piaA* (*piaBCD*

533   operon) (Hava and Camilli, 2002). These iron uptake systems have been demonstrated to be

534   important for full pneumococcal virulence, especially upon simultaneous mutation of two

535   operons (Brown *et al.*, 2001; Brown *et al.*, 2002). Single mutation of *piuB* resulted in only a

536   mild reduction of virulence in a pneumonia model, while no attenuated phenotype was

537   observed in systemic infection (Brown *et al.*, 2001).

538   Several studies have indicated a role for the Ami-AliA/AliB permease, encoded by the

539   *amiACDEF* operon and the *aliA* and *aliB* genes, in pneumococcal virulence. Mutants in *amiA*

540   and *amiC* displayed diminished adherence to pulmonary epithelial cells *in vitro* (Cundell *et*

541   *al.*, 1995), and *aliB* and *amiACD* were identified in STM screens of pneumococcal pneumonia

542   in a serotype 3 and 4 background, respectively (Lau *et al.*, 2001; Hava and Camilli, 2002).

543   Furthermore, using a collection of *aliA*, *aliB* and *amiA* single or triple mutants, the Ami-

544   AliA/AliB permease was shown to be required for successful nasopharyngeal colonization,

545   but not for pneumococcal pneumonia, with the most pronounced phenotype for the triple and

546   the *aliA* and *amiA* single mutants (Kerr *et al.*, 2004).

547   Inactivation of *codY* was previously reported to result in reduced adherence to

548   nasopharyngeal cells and reduced colonization in a mouse model of pneumococcal infection

549   (Hendriksen *et al.*, 2008). However, no significant differences in bacterial loads between wild

550   type and the *codY* mutant were observed in pneumonia and bacteremia models of infection. In

551   light of the above, we consider it unlikely that the truncation of *fatC* contributed to the

552   virulence phenotype associated with *codY* inactivation. On the other hand, mutation of *amiC*

553   may, at least partially, have been responsible for the observed attenuation during colonization,

554   but further experiments are needed to exactly determine the impact of the *amiC* mutation on

555   the virulence phenotype of the *codY* mutant.

556

557

558   *Why is CodY essential in* S. pneumoniae*?*

559

560   The finding that CodY is essential suggests that this regulator controls genes/functions that

561   are crucial for *S. pneumoniae*. The demonstration that mutations in *fatC* and *amiC* fully

562   restore viability of *codY* mutant cells may provide some clue as to these functions. As the

563   genome sequence analysis of D39Δ*codY* strongly suggested that the *fatC* mutation arose first

564   during establishment of the *codY* mutant construct, it is tempting to speculate about a possible

565   toxicity of iron in cells lacking CodY. If CodY is a repressor of the *fatD-fatC-fecE-fatB*

566   operon as previously concluded (Hendriksen *et al.*, 2008), derepression of iron uptake in its

567   absence may lead to accumulation of toxic concentrations of iron within the cell, thus creating

568   a strong selection pressure for the emergence of *fat* mutants. In this context, what could

569   account for the concomitant accumulation of *amiC* mutations? Considering only Ami and

570   CodY, it could be speculated that *codY* inactivation leading to derepression of the many

571   transporters of aa controlled by CodY and of Ami results in lethal imbalance of aminoacid

572   pools. However, this explanation is not readily connected to the co-occurrence of *fatC*

573   inactivation. In search of a possible direct connection, we came across the observation that the

574   housekeeping dipeptide permease of *Escherichia coli* allows utilization of heme as an iron

575   source (Letoffe *et al.*, 2006). Heme utilization has thus been shown to require a permease

576   made up of DppBCDF (dipeptide inner membrane transporter) as the ABC transporter and

577   either MppA (periplasmic L-alanyl-γ-D-glutamyl-meso-diaminopimelate binding protein) or

578   DppA (periplasmic dipeptide binding protein) as the substrate-binding protein. As *S.*

579   *pneumoniae* is devoid of a Dpp system, it is tempting to speculate that the Ami-Obl

580   oligopeptide transporter homologous to Dpp could allow heme utilization as an iron source.

581   The simultaneous involvement of CodY in *ami-obl* and *fat-fec* repression, and in *dpr*

582   activation would, upon *codY* inactivation, generate a severe oxidative stress because of the

583   simultaneous derepression of iron uptake via two transporters and depletion of the iron

584   storage-peroxide resistance Dpr protein (Fig. 7), hence the observed accumulation of *fat* and

585   *ami* mutations in the Δ*codY* background. It may not be a mere coincidence that the *ami* and *fat*

586   mutations which restore viability of *codY* mutant cells lead to competence (X-state)

587   derepression (Fig. 5 and Fig. S5). Since X-state is considered a pneumococcal substitute for

588   SOS (Claverys *et al.*, 2006; Prudhomme *et al.*, 2006), its induction may counteract oxidative

589   stress in *codY* mutant cells. Further investigation should reveal whether induction of the X-

590   state is required for tolerance of *codY* inactivation.

591   Transcriptome analysis revealed a striking parallel between CodY and RitR, an orphan

592   two-component signal transduction response regulator. Increased transcription of *piuB* and

593   *piuA* (i.e. *fatD* and *fatB*), as well as of *amiC* and decreased expression of *dpr* were reported in

594   *ritR* mutant cells (Ulijasz *et al.*, 2004) suggesting that CodY and RitR share these targets (Fig.

595   7). RitR was shown to bind three sites (RRB1-3; Fig. 6A) in the promoter region of the *fat*

596   operon (Ulijasz *et al.*, 2004). Recently, regulation of RitR binding at the *fat* promoter by a

597   Ser-Thr kinase-phosphatase, StkP-PhpP, was documented (Ulijasz *et al.*, 2009). While RitR

598   and PhpP jointly participate in complex formation at the *fat* promoter *in vitro*, addition of

599   StkP was shown to disrupt the complexes (Ulijasz *et al.*, 2009). This finding was consistent

600   with DNA microarray analyses of transcripts from an *stkP* knock-out showing dependence of

601   *fat* expression on StkP (Saskova *et al.*, 2007). The overlap between the CodY box

602   (AATTGTCAGAAATT located 3 nucleotides upstream of the -35 promoter box) and the first

603   RitR box (Fig. 6A) suggests that CodY may also interfere with RitR binding and adds a

604   degree to the complexity of *fat* regulation. CodY may thus represent one of the additional *fat*

605   regulators, the existence of which was proposed to account for the failure to detect the

606   opposite effects predicted for the individual deletion of *phpP* and *stkP* (Ulijasz *et al.*, 2009).

607   In line with the oxidative stress hypothesis (Fig. 7), *ritR* mutant cells were shown to display

608   greatly increased susceptibility to streptonigrin, which requires the presence of intracellular

609   iron, as well as to hydrogen peroxide (Ulijasz *et al.*, 2004). Iron overload due to derepression

610   of *fat* could be responsible for the latter by increasing intracellular free iron concentration and

611   therefore the potential for synthesis of reactive oxygen intermediates. If both CodY and RitR

612   are important for iron homeostasis and the reason for CodY essentiality is to prevent oxidative

613   stress, what about the viability of *ritR* mutant cells? As there was no mention of difficulty in

614   generating and/or growing *ritR* mutants, CodY might be more important for *ami*, *fat* and *dpr*

615   regulation than RitR. Alternatively, RitR and CodY could be equally important and the

616   problematic viability of *ritR* mutant cells has been overlooked. It would therefore be

617   interesting to check whether a previously constructed *ritR* mutant is readily transferred by

618   transformation.

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620

621   *Concluding remarks*

622

623   Further work with other clinical isolates of *S. pneumoniae* is necessary to establish whether

624   the essentiality of CodY is a general feature of this species. To the best of our knowledge, this

625   is the first report in any bacterium that a member of the CodY family is essential. Is this

626   situation unique to *S. pneumoniae*? It would be interesting to investigate whether CodY is

627   essential in species closely related to *S. pneumoniae* and in streptococci in general. It is

628   possible that CodY is also essential in other species but that the presence of suppressor

629   mutation(s) has been overlooked. Our observations may thus prompt careful reexamination of

630   the viability of the *codY* mutants previously constructed in other species. More generally, the

631   above described tests of acceptance frequencies by transformation should be used routinely

632   when working with 'important' genes, to prevent the presence of suppressors going

633   undetected in the future. In any case, because of its essentiality in a major human pathogen,

634   CodY constitutes a potentially interesting new therapeutic target.

635

636

Experimental procedures

638

Bacterial strains, culture and transformation conditions

640

S. pneumoniae strains and plasmids used in this study are described in Table 1. Stock cultures

were routinely grown at 37°C in Todd-Hewitt plus yeast extract (THY) medium to OD_{550}=0.3;

after addition of 15% glycerol, stocks were kept frozen at -70°C. To investigate spontaneous

competence development, cells were gently thawed and aliquots were inoculated (1 into 25) in

C+Y. The initial pH value was adjusted to 7.0 and trypsin (2 μg ml^{-1}) was added to prevent

spontaneous competence induction in the preculture. After incubation at 37°C to OD_{550}=0.2,

cultures were centrifuged and cells were concentrated to OD_{550} of 0.4 in fresh medium

containing 15% glycerol and kept frozen at -70°C. For the monitoring of growth and

spontaneous competence development, these precultures were gently thawed and aliquots

were inoculated (1 into 50, unless otherwise indicated) in luciferin-containing C+Y medium

and distributed into a 96-well microplate (300 μl per well). Measurement of competence

involved the use of an *ssbB*::*luc* transcriptional fusion which reports on competence through

light emission by luciferase (Prudhomme and Claverys, 2007). RLU (relative luminescence

unit) and OD values were recorded throughout incubation at 37°C (in a Varioskan Flash

luminometer; Thermo Electron Corporation).

CSP-induced transformation was performed as described previously (Martin *et al.*, 2000),

using precompetent cells treated at 37°C for 10 min with synthetic CSP1 (100 ng ml^{-1}). After

addition of transforming DNA, cells were incubated for 20 minutes at 30°C. Transformants

were selected by plating on CAT-agar supplemented with 4% horse blood, followed by

660   challenge with a 10 ml overlay containing chloramphenicol (4.5 µg ml^{-1}), erythromycin (0.05

661   µg ml^{-1}), kanamycin (250 µg ml^{-1}), methotrexate (2.2 µg ml^{-1}), spectinomycin (100 µg ml^{-1}),

662   streptomycin (200 µg ml^{-1}) or trimethoprim (20 µg ml^{-1}), after phenotypic expression for 120

663   min at 37°C.

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665

666   *Mutagenesis and duplication of* codY

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668   Insertions of *kan* (Kan^R) or *spc* (Spc^R) minitransposons were generated by *in vitro mariner*

669   mutagenesis as described (Prudhomme *et al.*, 2007). Plasmids used as a source for the

670   minitransposons were pR410 and pR412, respectively (Table 1). Briefly, plasmid DNA (~1

671   µg) was incubated with a target PCR fragment (indicated in the legend of Fig. 1) in the

672   presence of purified *Himar1* transposase, leading to random insertion of the minitransposon

673   within the fragment. Gaps in transposition products were repaired as described (Prudhomme

674   *et al.*, 2007) and the resulting *in vitro*-generated transposon insertion library was used to

675   transform *S. pneumoniae*. Location and orientation of minitransposon insertions were

676   determined as previously described (Prudhomme *et al.*, 2007) through PCR reactions using

677   primers MP127 or MP128 in combination with either one of the primers used to generate

678   *codY* PCR fragments (Table 1). Cassette-chromosome junctions were sequenced for some

679   insertions as indicated in the legend of Fig. 1.

680   Placement of a second copy of *codY* under the control of the maltose-inducible P$_M$

681   promoter at CEP was achieved by cloning into *Nco*I-*Bam*HI digested pCEP2 plasmid DNA a

682   *codY* PCR fragment generated using the codYatg-codYstop primer pair (Table 1) and digested

683   with *Bam*HI and *Nde*I. The resulting recombinant plasmid pCEP-codY was used as donor in

684   transformation of strain R1501 followed by selection for a Kan^R transformant, thus generating

685   strain R2349 (Table 1). Plasmid pCEP2 was generated in this study as a high copy number

686   derivative of plasmid pCEP (Guiral *et al.*, 2006). Briefly, an *Eco*RI-*Pst*I fragment from pCEP

687   was ligated to *Eco*RI-*Pst*I digested pKL147 (Table 1) to replace the pSC101 replication

688   machinery of pCEP by the pBR replication machinery and Ap^R resistance gene of pKL147.

689

690

691   *Reversion of* codY *knock-out by transformation*

692

693   To replace *codY*::*trim* or *codY*::*spc* insertions by *codY*$^+$, we took advantage of the *kan*^{90C}

694   *mariner* insertion. This insertion is located immediately upstream the CodY binding site

695   (CYB) in the *codY* promoter region (Fig. 1A) and does not inactivate *codY*; we used it as a

696   marker to select for the re-introduction of the *codY*$^+$ gene by co-transformation with Kan^R.

697   To allow the use of a similar strategy for replacement of *codY*::*trim* in *kan*^{R} strains (such as

698   TD83), the *kan*^{90C} cassette was exchanged with the *spc*^{R} cassette by transformation of strain

699   R2641 with plasmid pR412 DNA, selecting for Spc^R transformants to generate strain R2644

700   (genotype referred to as *spc*^{90C}::*codY*$^+$; Table 1). The exchange is based on the presence of

701   DNA homology at the borders of the synthetic *spc* and *kan* minitransposons, allowing

702   exchange of the resistance cassette genes by homologous recombination during

703   transformation.

704

705

706   *Whole genome sequencing of D39 and D39∆codY*

707

708   Roche 454 FLX whole genome sequencing was performed by Agowa Genomics (Berlin,

709   Germany) using genomic DNA isolated from mid-log cultures by the Genomic DNA kit

710   (Qiagen). For each strain, a shotgun library and a 3-kb span paired end library were generated

711   according to Roche standard protocols, mixed in equal parts (about 400,000 beads from each

712   library) and sequenced using default settings on a ¼ picotiterplate. A total of 240496 reads of

713   which 65336 contained paired ends were obtained for D39Δ*codY* (29-fold coverage), and

714   210631 reads with 60676 paired ends were obtained for D39 (25-fold coverage). *De novo*

715   assembly was carried out using the Roche 454 Newbler software (Release 2.3

716   (091027_1459), resulting in 68 contigs in 5 scaffolds for D39Δ*codY*, and 85 contigs in 5

717   scaffolds for D39.

718   Data from the sequencing runs were mapped to the reference D39 strain (Acc.no.:

719   NC_008533.1) and the variations thereto scored using the Roche 454 Reference Mapper

720   software [Release 2.3 (091027_1459)].

721

722

723   *Sequencing of* amiC *and* fatC *of individual D39Δ*codY *clones*

724

725   The *amiC* and *fatC* sequence of the D39Δ*codY* population was verified by a PCR-sequencing

726   approach. To this end, chromosomal DNA was isolated from individual clones by cetyl-

727   trimethylammonium bromide (CTAB) extraction as described previously (van Soolingen *et*

728   *al.*, 1994). The *amiC* and *fatC* loci were PCR-amplified under standard conditions using,

729   respectively, primers HBDamiCF1 and HBDamiCR2 and HBDfatCF and HBDfatCR (Table

730   1). Subsequently, both strands were sequenced using the same primers used for PCR as well

731   as internal primers HBDamiCR1 and HBDamiCF2 (Table 1) in case of *amiC*.

732

733

Acknowledgements

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744   **Supplementary material**

745

746   The following Supplementary Material is available for this article:

747   **Fig. S1.** Attempt at depleting CodY and consequences on growth and competence in C+Y
748   medium.

749

750   **Table S1.** Variations between the D39 NCTC 7466 isolate sequenced in this study and the
751   previously published D39 NCTC 7466 genome sequence (Lanie *et al.*, 2007).

752

753   **Fig. S2.** Proposed unique mutational event responsible for the $amiC^{G246TT}$ $amiC^{A248C}$ (or
754     $amiC^{GGA \rightarrow TTGC}$) changes.

755

756   **Fig. S3.** Localization of *ami* mutation in strain D39Δ*cps*Δ*codY* via transformation.

757

758   **Table S2.** The original D39Δ*codY* strain displays a mixed population structure with respect to
759   *amiC*.

760

761   **Fig. S4.** Distribution of *spc mariner* minitransposon insertions in the *fat-fec* operon.
762

Fig. S5. Effect of *ami*, *fat* and *ami fat* inactivation on growth and spontaneous competence development.

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918   **Table 1.** Strains, plasmids, and primers used in this study.

919

Strains	Genotype[a]/description	Source/reference
Streptococcus pneumoniae		
D39	Serotype 2	NCTC 7466
D39Δ*codY*	D39 Δ*codY*::*trim* (*socY**)[b]; Trim[R], (Mtx[R])[b]	(Hendriksen *et al.*, 2008)
D39Δ*cps*	D39 Δ*cps*::*kan*; Kan[R]	(Hendriksen *et al.*, 2008)
D39Δ*cps*Δ*codY*	D39 Δ*cps*::*kan* Δ*codY*::*trim* (*socY*)[c]; Kan[R], Trim[R], (Mtx[R])[c]	(Hendriksen *et al.*, 2008)
R6	Unencapsulated derivative of D39	(Burghout *et al.*, 2007)
R246	R800 but *hexAΔ3*::*ermAM*; Ery[R]	(Mortier-Barrière *et al.*, 1998)
R304	R800 derivative, *nov1*, *rif23*, *str41*; Nov[R], Rif[R], Sm[R]	(Mortier-Barrière *et al.*, 1998)
R800	R6 derivative	(Lefèvre *et al.*, 1979)
R895	R800 but *ssbB*::*luc* (*ssbB*[+]); Cm[R]	(Chastanet *et al.*, 2001)
R1501	R800 but *comC*₀	(Dagkessamanskaia *et al.*, 2004)
R1818	R1501 but *hexAΔ3*::*ermAM*; Ery[R]	This study
R2349	R1501 but CEP_M-*codY* (the resulting duplication of *codY* is denoted *codY*^{+/+}); Kan[R]	This study
R2350	R895 but *codY*^{+/+}; Kan[R], Cm[R]	This study
R2424	R2350 but genuine *codY* inactivated by *mariner* insertion *spc*[3A] (the resulting *codY* combination is denoted *codY*[spc3/+]); Kan[R], Cm[R], Spc[R]	This study

R2425	R2350 but ectopic *codY* inactivated by *mariner* insertion *spc*[3A] (the resulting *codY* combination is denoted *codY*[+/spc3]); Kan[R], Cm[R], Spc[R]	This study
R2427	R6 but *codY*^{+/+}; Kan[R]	This study
R2428	R1818 but *codY*^{+/+}; Ery[R], Kan[R]	This study
R2430	R895 but p*malR* (pAPM22); Cm[R], Ery[R]	This study
R2432	R2424 but p*malR* (pAPM22) ; Cm[R], Ery[R], Kan[R],Spc[R]	This study
R2437	R2349 but *str41*; Kan[R], Str[R]	This study
R2438	R2437 but *codY*[spc3/+]; Kan[R], Spc[R], Str[R]	This study
R2549	R895 but *amiC9*; Cm[R], Mtx[R]	This study
R2641	R895 but *kan*[90C]::*codY*[+] , *str41*; Cm[R], Spc[R], Str[R]	This study
R2644	R2641 but *spc*[90C]::*codY*[+]; Cm[R], Spc[R], Str[R]	This study
R2737	R895 but CEP$_M$; Cm[R], Kan[R]	This study
R3002	R246 but *fatC*::*cat*[23C] (from TD131); Cm[R], EryR	This study
R3003	R246 but *amiC9*; Ery[R], Mtx[R]	This study
R3004	R3002 but *amiC9*; Cm[R], Ery[R], Mtx[R]	This study
TD73	D39 but *codY*^{+/+}; Kan[R]	This study
TD80	D39Δ*cps*Δ*codY* (*socY*)[b] but *str41*; Kan[R], Str[R], Trim[R]	This study
TD81	TD80 but *codY*::*spc*[3A]; Kan[R], Spc[R], Str[R]	This study
TD82	D39Δ*cps* but *ssbB*::*luc* (*ssbB*[+]); Cm[R], Kan[R]	This study
TD83	D39Δ*cps*Δ*codY* but *ssbB*::*luc* (*ssbB*[+]); Cm[R], Kan[R], Trim[R]	This study
TD84	TD80 but *amiC9*; Cm[R], Kan[R], Mtx[R]	This study
TD95	TD83 but *spc*[90C]::*codY*[+]; Cm[R], Kan[R], Spc[R]	This study
TD96	TD95 but *codY*[+]; Cm[R], Kan[R]	This study
TD129	D39Δ*cps* but *fatC*::*spc*[23C]; Kan[R], Spc[R]	This study
TD130	TD129 but *amiC*9; Kan[R], Mtx[R], Spc[R]	This study
TD131	TD129 but *fatC*::*cat*[23C]; Cm[R], Kan[R]	This study
TD135	TD130 but *ssbB*::*luc* (*ssbB*[+]); Cm[R], Kan[R], Mtx[R], Spc[R]	This study

| TD138 | TD129 but *ssbB*::*luc* (*ssbB*+); Cm[R], Kan[R], Spc[R] | This study |
| TD154 | TD135 but Δ*codY*::*trim*; Cm[R], Kan[R], Mtx[R], Spc[R], Trim[R] | This study |

Plasmids

pAPM22	pLS1 derivative carrying the *malR* gene; Ery[R]	(Puyet *et al.*, 1993)
pCEP	pSC101 derivative (i.e. low copy number plasmid) carrying CEP; Spc[R], Kan[R]	(Guiral *et al.*, 2006)
pCEP2	pKL147 derivative (i.e. high copy number plasmid) carrying an *Eco*RI/*Pst*I fragment from pCEP; Spc[R], Kan[R], Ap[R]	This study
pCEP2-*codY*	pCEP2 derivative carrying *codY* under P$_M$ control; Spc[R], Kan[R], Ap[R]	This study
pEM*cat*	ColE1 derivative carrying a Cm[R] *mariner* minitransposon; Ap[R], Cm[R]	(Akerley *et al.*, 1998)
pKL147	pUS19 derivative containing *gfpmut2* fused to the 3' end of *dnaX* with a linker; Spc[R], Ap[R]	(Lemon and Grossman, 1998)
pR410	pEM*cat* derivative carrying a Kan[R] (*kan* gene) *mariner* minitransposon; Ap[R], Kan[R]	(Sung *et al.*, 2001)
pR412	pEM*cat* derivative carrying a Spc[R] (*aad9* gene, also called *spc*) *mariner* minitransposon; Ap[R], Spc[R]	(Martin *et al.*, 2000)

Primers	**Sequence[d]; gene; position[§]**	
ami1	GCGCAAACAGGCTCTAAGGG; *amiA*; +1815	This study
ami2	TCAGGAATTCCTGCTGCCATTAT; *amiC*; +1257	This study
ami4	CCTGACTCACCTACCAAGGCTA; *amiD*; +712	This study
ami5	CCTTCACCGAAGGAAATTTCTA; *amiE*; +121	This study
ami6	TTAGCTGACTTCAACCCACTACA; *amiF*; +1027	This study
amiF1	GCCTTGCTTTCAGCGGTACCAAT; *amiF*; +789	This study

39

AM40	AGAGTTTCGGATGGTTTGGA; *treR*; +347	This study
codY1	CAAGGATCAGTTTTCCCATATTTTCG; *codY*; +1636	This study
codY2	CTTCGTGTCCTTCGTGACTTTA; *codY*; -1004	This study
codYatg	tgaatc**ATG**aCACATTTATTAGAAAAAACTAG; *codY*; 0	This study
codYstop	aaattggatccTTTGTCA**TTA**GTAATCTCTTTTC; c*odY*; +797	This study
fat1	GCGAACGAATGATTTACTGG; *fatD*; -659	This study
fat2	TCTCACCAGTCTTTCCACCC; *fatB*; +1388	This study
fatC1	TAAAAGCAAACATACCAAGC; *fatC*; -9	This study
fatC2	TAAAGAATAAGAAGCCACCC; *fatC*; +909	This study
HBDamiCF1	ACGGCTGATAAACGTGATAA; *amiC*; +145	This study
HBDamiCF2	GTCGTTGGTCTTGTCTTCAT; *amiC*; +1381	This study
HBDamiCR1	TAAATTCTCCCAAAGTCCAA; *amiC*; +343	This study
HBDamiCR2	CGCATCAATAGTTTCAGAGG; *amiC*; +1568	This study
HBDfatCF	ACACTGATGAAGCAAGACCT; *fatC*; +376	This study
HBDfatCR	CAATATCTGAGCCGTTTCTC; *fatC*; +645	This study
kan1	ATCATGTCCTTTTCCCGTTCCAC; *kan*; +191	This study
MP127	CCGGGGACTTATCAGCCAACC; mariner transposon	(Martin *et al.*, 2000)
MP128	TACTAGCGACGCCATCTATGTG; mariner transposon	(Martin *et al.*, 2000)
MP188	TTCATTTTCACCAACCAGGTTAC; *codY*; +1032	This study
MP189	ATTGGCTGCTGAGTTTACTCCAG; *codY*; -618	This study

MP192	ggatccACGTCATCAACTAAATAGCG; *aliA*; -343	This study
MP193	CAGAAGCTTTCTGGTTTGTT; *aliA*; -539	This study
MP194	TTGGAATTCCCTCTTCTGGAAC; *dexB*; +908	This study
MP195	ttagttgatgacgtggatccGCTTTTTATACAGTCCTCCC; *dexB*; +1693	This study
rpsL_3	TGACATGGATACGGAAGTAG; *rspL*; -798	This study
rpsL_4	ATGGTAAGCTGAGTTATAGC; *rpsL*; +1204	This study

920

921 [a] Ap[R], ampicillin resistance; Cm[R], chloramphenicol resistance; Kan[R], kanamycin resistance;

922 Mtx[R], methotrexate resistance; Spc[R], spectinomycin resistance; Str[R], streptomycin resistance;

923 Trim[R], trimethoprim resistance.

924

925 [b] *socY** refers to the presence of the *amiC23*, *amiC^{A248C}* or *amiC^{G1459T}* mutations as well as the

926 *fatC^{C496T}* identified in this study and demonstrated to be required for tolerance of *codY*

927 inactivation.

928

929 [c] *socY* refers to the presence of the *amiC^{G1459T}* and *fatC^{C496T}* mutations.

930

931 [d] Lowercase letters indicate nucleotide extensions to introduce convenient restriction sites

932 (*Nde*I, *Bam*HI, and *Eco*RI, respectively in codYatg, codYstop, and MP192; underlined

933 sequences) in the primers [Note that the *Nde*I site in codYatg introduced a Ala → Thr change

934 at the second aa position of CodY]. The start and stop codons of *codY* are shown in bold. In

935 MP195, lowercase letters indicate nucleotide extension complementary to MP192.

936

937   §Position is given with respect to the ATG of the corresponding gene; - and + indicate

938   upstream and downstream, respectively.

939

940   C and A indicate respectively the co-transcribed and the reverse orientation of an inserted mini-

941   transposon antibiotic resistance cassette with respect to the targeted gene.

942   **Fig. 1.** Insertion mutagenesis of *codY* is not possible in *S. pneumoniae* R6 unless the strain

943   carries an ectopic complementing copy of the gene.

944   A. Distribution of *mariner* insertions in the MP188-MP189 fragment obtained in wild type

945   cells.

946   B. Placement of a second copy of *codY* under the control of the maltose-inducible P_M

947   promoter at CEP.

948   The inset in (B) shows control PCRs with the primer pair amiF1-AM40: lane 1, 2083 bp-long

949   wildtype fragment; lane 2, 2760 bp-long CEP_M-*codY-kan* fragment. M, kb ladder.

950   C Recovery of *mariner* insertions at the *codY* locus in a recipient harboring a complementing

951   ectopic copy.

952   The location of primers used to generate *codY* PCR fragments for *mariner* mutagenesis,

953   MP188-MP189 (A) and codY1-codY2 (C) is indicated. Insertion sites were located through

954   DNA sequencing at position -100 for kan^{90C} (A) and -32, +163, +168, +300, and +365 for

955   spc^{5A}, spc^{4C}, spc^{3A}, spc^{12A}, and spc^{8A}, respectively (C). Positions are given with respect to the

956   first nucleotide of *codY* taken as +1; ^{C} and ^{A} indicate co-transcribed and reverse orientation of

957   *spc* (*s*) or *kan* (*k*) cassette, respectively, with respect to *codY*. A CodY box

958   (AATTTTCAGATAATT) previously identified in the promoter region of *codY* (Hendriksen

959   *et al.*, 2008) is indicated as CYB. It is separated by 1 nt from the putative -35 promoter box of

960   *codY*, itself separated by 17 nt from an extended -10 box with perfect match to the consensus

961   (last nucleotide of this -10 located 26 nt upstream of the *codY* start).

962   **Fig. 2.** Integration of *mariner* minitransposons can occur at either site in the chromosome of

963   of *codY*$^{+/+}$ recipient cells.

964   A. PCR probing of the structure of an Spc^R transformant (strain R2424) carrying the

965   *codY*::*spc*^{3A} minitransposon at the genuine *codY* locus using the following primer pairs:

966   codY1-codY2 (fragment/lane 2, 3787 bp), codY2-MP127 (fragment/lane 4, 1493 bp), MP127-

967   codY1 (fragment/lane 3, 1196 bp), and amiF1-AM40 (fragment/lane 5, 1684 bp). Control

968   PCR of the parental structure (strain R2350) using primer pairs codY1-codY2 (fragment/lane

969   1*, 2641 bp) and amiF1-kan1 (fragment/lane 6*, 1684 bp).

970   B. PCR probing of the structure of an Spc^R transformant (strain R2425) carrying the

971   *codY*::*spc*^{3A} minitransposon at the CEP-*codY* locus using the following primer pairs: codY1-

972   codY2 (fragment/lane E, 2641 bp), amiF1-kan1 (fragment/lane B, 2830 bp), amiF1-MP127

973   (fragment/lane C, 815 bp), and MP127-kan1 (fragment/lane D, 917 bp). Control PCR of the

974   parental structure (strain R2350) using primer pairs codY1-codY2 (fragment/lane F*, 2641

975   bp) and amiF1-kan1 (fragment/lane A*, 1684 bp). M, kb ladder.

976

977

978   **Fig. 3.** Comparing transformation frequencies of *codY*$^{spc3/+}$ in wildtype and *codY*$^{+/+}$ recipient

979   cells.

980   Chromosomal DNA of strain R2438 (*codY*$^{spc3/+}$ *str41*) was used as donor. Strains used:

981   isogenic *codY*$^+$ and *codY*$^{+/+}$ derivatives of R800, R6, and D39, respectively R895 and R2350,

982   R6 and R2427, and D39 and TD73.

983

984

44

Fig. 4. Transformation data indicate the presence of two independent suppressor mutations or a chromosomal rearrangement in the previously described *codY* mutant. Chromosomal DNA of strain TD81 (*codY*::*spc*^{3A} *socY str41*) was used as donor. Strains used: isogenic *codY*$^+$ and *codY*$^{+/+}$ derivatives of R800-*hex*$^+$, R6, D39, and R800-*hex*$^-$, respectively R895 and R2350; R6 and R2427; D39 and TD73; R1818 and R2428.

Fig. 5. Effect of *codY* inactivation and of *socY* on spontaneous competence development. Pre-cultures prepared as described in the Experimental procedures were inoculated (1/50th dilution) in C+Y medium (containing 300 μg ml^{-1} glutamine) and competence (black symbols and curves) and OD (grey symbols and curves) values were recorded throughout incubation at 37°C. Values correspond to individual cultures representative of three independent experiments. Strains used: *codY*$^+$ TD82 (panels A-B); *codY socY* mutant TD83 (panels C-D); *socY* mutant TD96 (panels E-F).

Fig. 6. Inactivation of *ami* and *fat* results in tolerance of *codY* inactivation.

A. Distribution of *fatC* and *amiC* mutations identified in strain D39Δ*codY* by whole genome sequence comparison. Positions are given with respect to the first nucleotide of *fatC* (top) and *amiC* (bottom), respectively, taken as +1. The location of the *fatC*::*spc*^{23C} *mariner* insertion and *amiC9* mutation used in panel B is also indicated.

CYB, CodY binding sites identified in the promoter region of the *fat-fec* and *ami* operons (Hendriksen *et al.*, 2008); -35 and -10 promoter boxes; RRB1-3, RitR binding sites identified

1008   in front of the *fatD* (also called *piuB*) gene (Ulijasz *et al.*, 2004). ABC, ATP-binding cassette

1009   protein, MP, membrane protein, SBP, substrate binding protein.

1010   B. The *fatC amiC* combination of mutations allows tolerance of *codY* inactivation.

1011   Chromosomal DNA of strain TD80 (*codY*::*trim str41*) and TD81 (*codY*::*spc*[3A] *str41*) was used

1012   as donor and Str[R] as well as, respectively, Trim[R] and Spc[R] transformants were scored. Strains

1013   used: wt, R246; *fatC*::*cat*[23C] mutant, R3002; *amiC9* mutant, R3003; *amiC9-fatC*::*cat*[23C]

1014   double mutant, R3004.

1015

1016

1017   **Fig. 7.** Iron toxicity may account for inviability of *codY* mutant cells in *S. pneumoniae*.

1018   CodY and RitR share regulatory targets: CodY is a repressor of *ami*, *obl* and *fat-fec*, but

1019   activates *dpr*, which encodes an iron-storage peroxide resistance protein (Hendriksen *et al.*,

1020   2008); RitR directly represses *fatD* (or *piuB*) and inactivation of *ritR* derepresses *amiC* while

1021   reducing *dpr* expression (Ulijasz *et al.*, 2004). We propose that inactivation of *codY* is not

1022   tolerated because it results in simultaneous derepression of iron uptake via Fat-Fec and

1023   possibly of heme utilization via Ami (see Discussion), as well as depletion of Dpr, which is

1024   required to protect cells from iron toxicity. ILV, isoleucine-leucine-valine (CodY activating

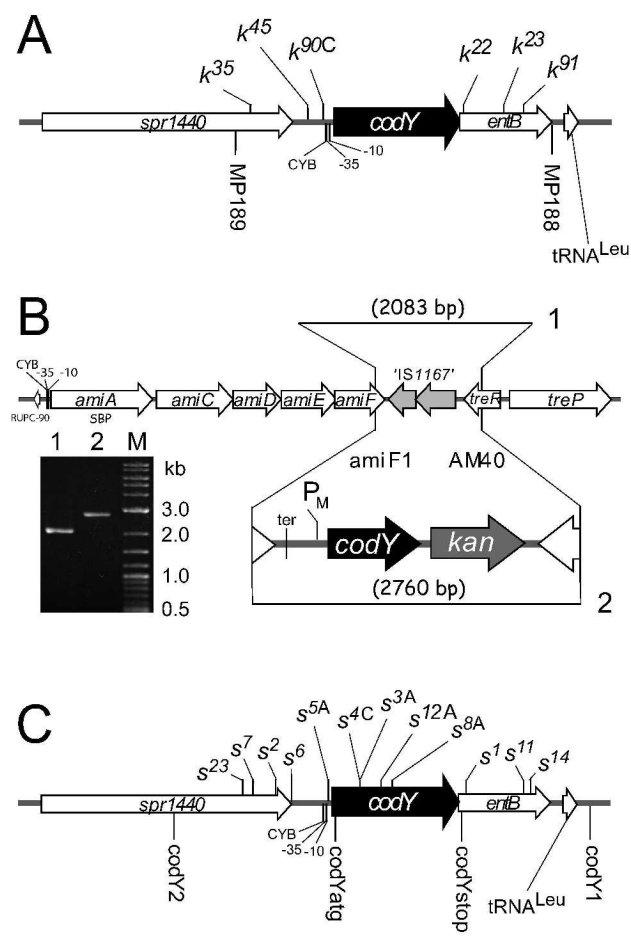1025   branched chain aminoacids).

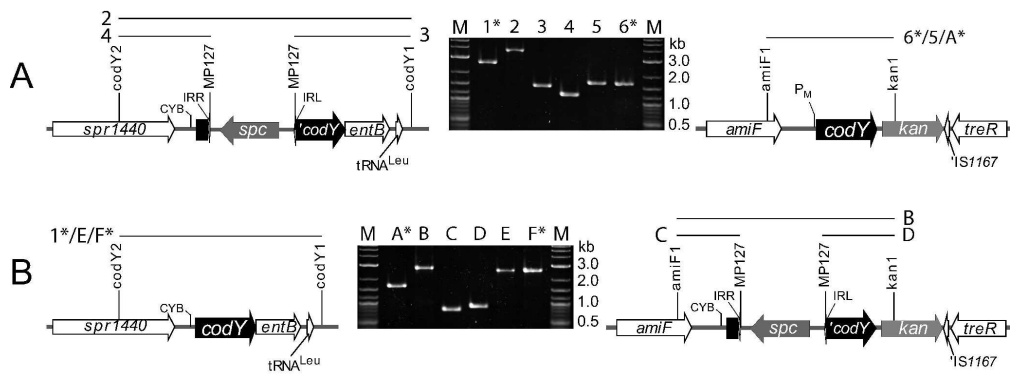FIG. 1. Caymaris *et al.*

109x158mm (600 x 600 DPI)

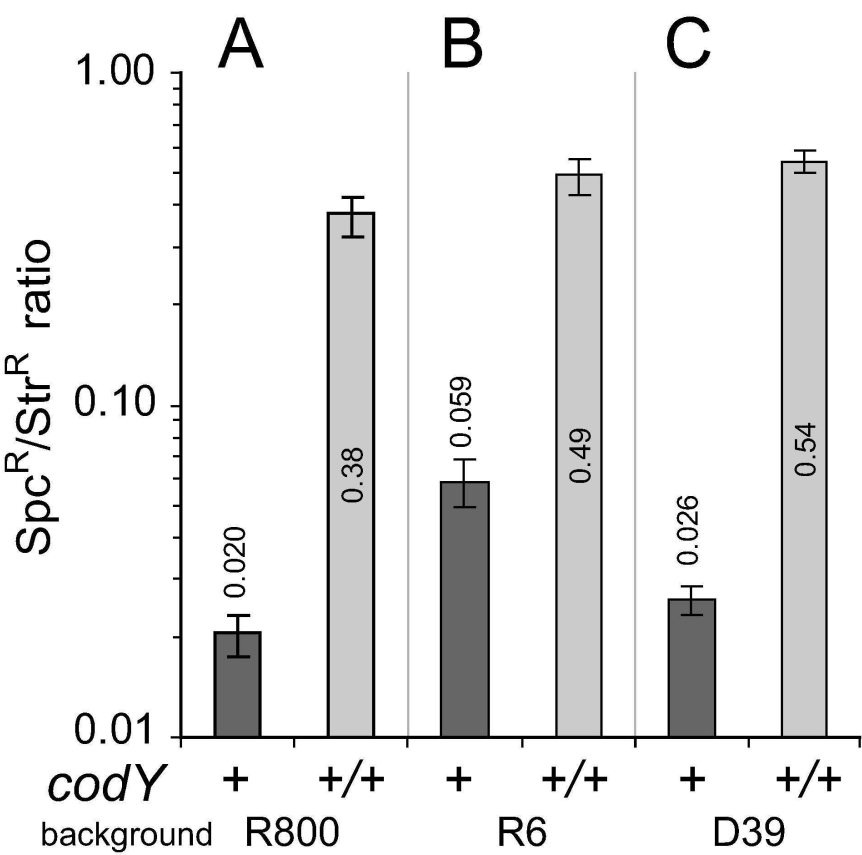FIG. 2. Caymaris *et al*.

173x98mm (600 x 600 DPI)

FIG. 3. Caymaris *et al*.
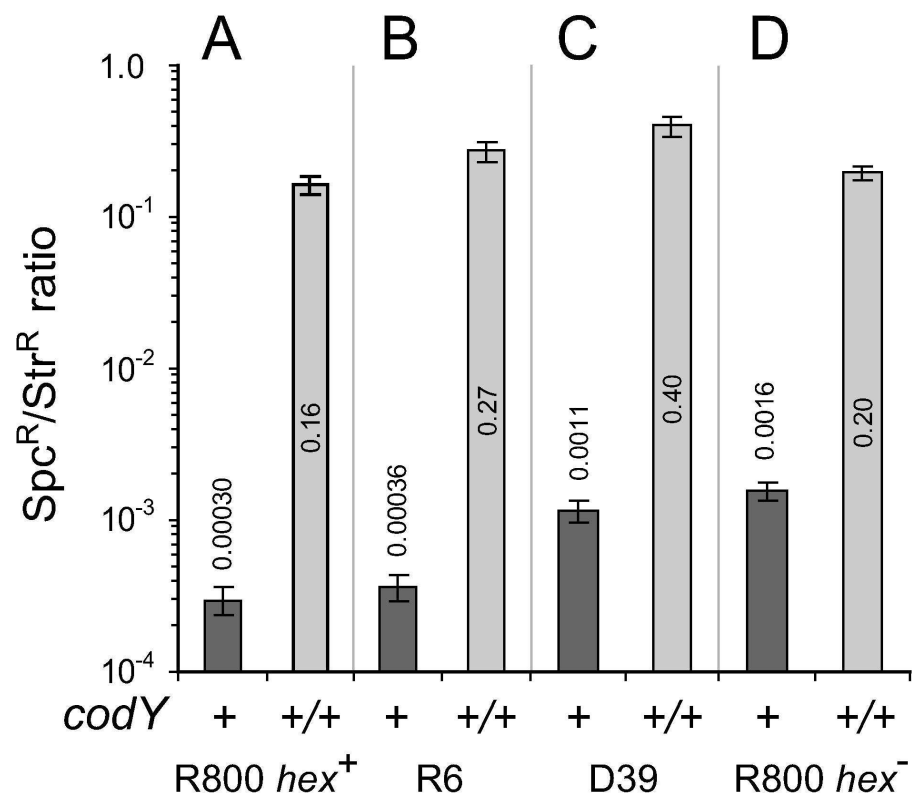
83x111mm (600 x 600 DPI)

FIG. 4. Caymaris *et al*.
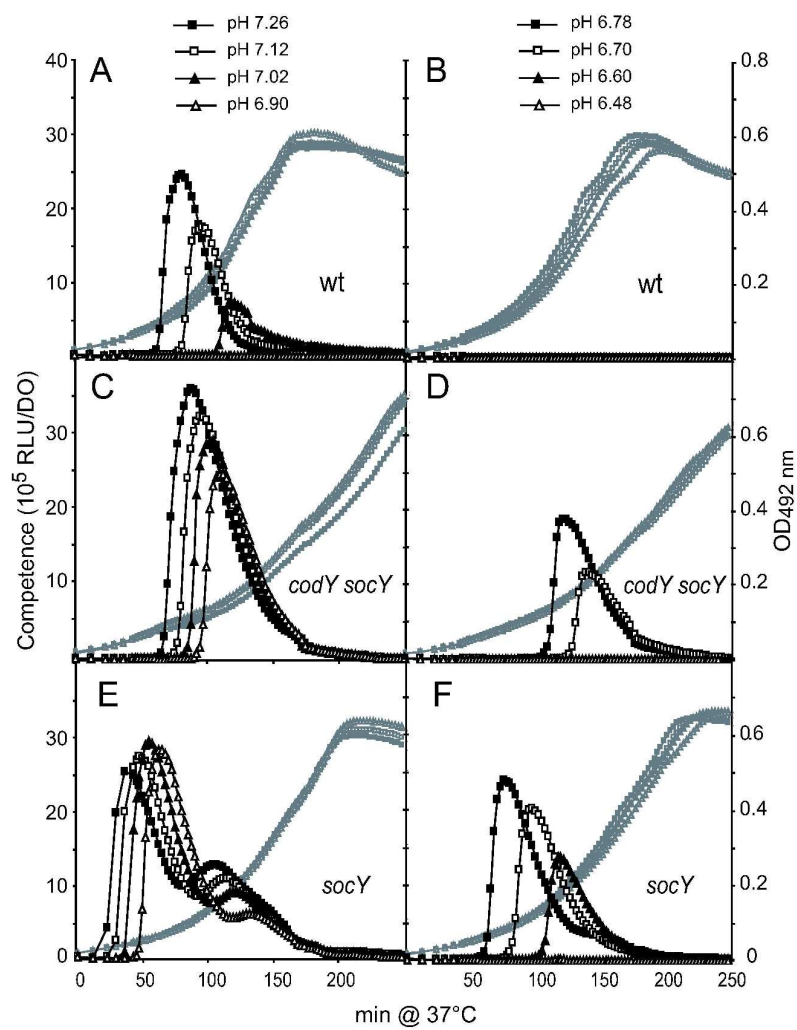
82x106mm (600 x 600 DPI)

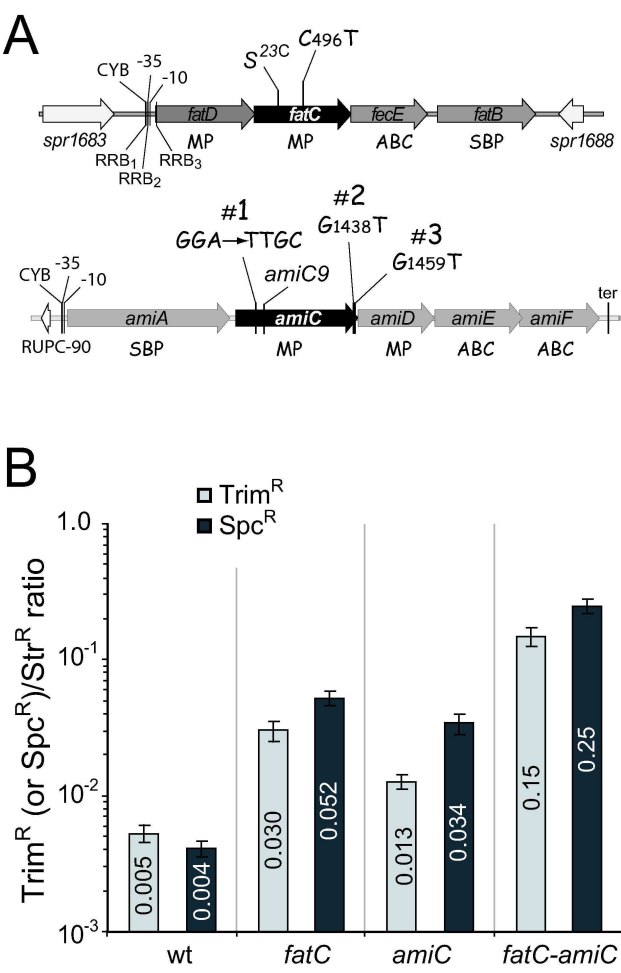FIG. 5. Caymaris *et al.*

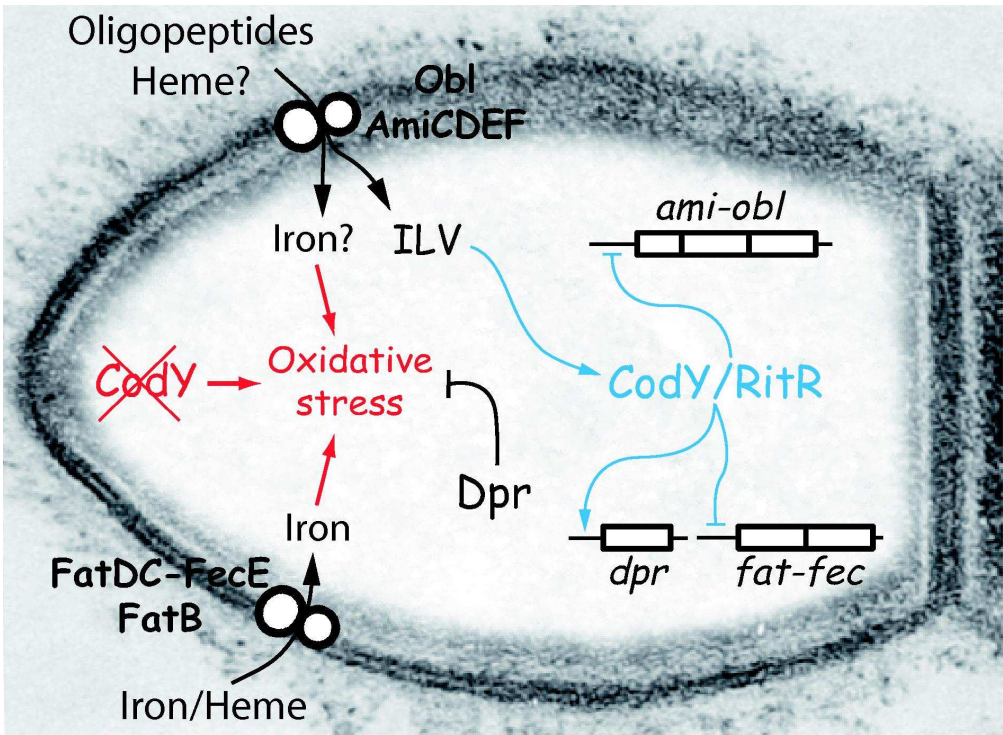111x168mm (600 x 600 DPI)

FIG. 6. Caymaris *et al*.

80x154mm (600 x 600 DPI)

FIG. 7. Caymaris *et al.*

80x86mm (600 x 600 DPI)