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

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Essentiality of CodY in *Streptococcus pneumoniae*

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Summary

CodY is a global regulator highly conserved in low-G+C Gram-positive bacteria. It plays a key role in the adaptation of *Bacillus subtilis* to nutritional limitation through repression of a large gene set during exponential growth and relief of repression upon starvation. In several pathogenic bacteria, CodY regulates major virulence genes. Our interest in *Streptococcus pneumoniae* CodY originates from our observations that the oligopeptide permease Ami was involved in repression of competence for genetic transformation. We hypothesized that peptide uptake through Ami feeds amino acid pools, which are sensed by CodY to repress competence. As our initial attempts at inactivating *codY* failed, we launched an in-depth analysis into the question of the essentiality of *codY*. We report that *codY* cannot be inactivated unless a complementing ectopic copy is present. We obtained genetic evidence that a recently published D39 *codY* knock-out contains additional mutations allowing survival of *codY* mutant cells. Whole genome sequencing revealed mutations in *fatC*, which encodes a ferric iron permease, and *amiC*. This combination of mutations was confirmed to allow tolerance of *codY* inactivation. The *amiC* mutation is in itself sufficient to account for the 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 Hävarstein, 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.
Exhaustion of nutrients would be sensed through a mechanism involving peptide uptake by the Ami-Obl oligopeptide permease, followed by peptidase digestion to release amino acids (aa) leading to replenishment of aa pools, which in turn affect a global regulatory protein such as CodY (Claverys et al., 2000). The above-mentioned demonstration that BCAAs are effectors of CodY in several species would fit nicely with this hypothesis since S. pneumoniae Obl proteins have different but overlapping specificities for peptides including those containing BCAAs (Alloing et al., 1994). Thus import of BCAAs (as part of oligopeptides) by the Ami-Obl permease could directly impact CodY activity. This situation would not be unprecedented since CodY-dependent repression of comK and srfA was lost in an opp (oligopeptide permease) mutant of B. subtilis (Serror and Sonenshein, 1996). To account for the negative impact of Ami-Obl on competence induction, we explicitly stated in our model that CodY should repress competence (Claverys et al., 2000).

To test this model, we initiated experiments aimed at inactivating S. pneumoniae codY. Surprisingly, this gene turned out to be difficult to inactivate. However, codY inactivation had recently been achieved through insertion of a trimethoprim resistance gene (trim) in strain D39 (Hendriksen et al., 2008). Microarray analysis using this mutant suggested that pneumococcal CodY functions mainly as a transcriptional repressor, as 43 of the 47 genes differentially expressed in the codY::trim mutant were found to be upregulated. The inefficiency in constructing a codY knock-out in strain R6 prompted us to launch an in-depth analysis of the question of the essentiality of codY. Here, we present our data establishing that codY is essential in our laboratory strains, as well as in S. pneumoniae R6 and encapsulated D39 strains. We provide genetic evidence that, in addition to the codY knock-out, the codY::trim mutant used for transcriptome analysis of the CodY regulon and for virulence studies (Hendriksen et al., 2008) contains additional mutations (suppressors) allowing survival of codY mutant cells. We document a strong derepression of competence in such
codY-mutant cells. Finally, we use comparative whole genome sequencing to identify suppressor mutations in fatC, which encodes a ferric iron permease, and amiC. We confirm that this combination of mutations allows tolerance of codY inactivation and discuss possible reasons for CodY essentiality in S. pneumoniae in light of these observations.

Results

Minitransposon insertion mutagenesis of S. pneumoniae codY is not possible

A codY PCR fragment amplified with the primer pair MP188-MP189 (Table 1) was used as target for mariner mutagenesis (Experimental procedures). Analysis of more than 50 minitransposon insertions through PCR revealed that only 6 clones produced an MP188-MP189 fragment of which the size was consistent with that predicted for integration of the kanR minitransposon (donated by plasmid pR410; Table 1). Localization of these 6 insertions indicated that none of them had inserted into the codY gene (Fig. 1A). Given our previous experience with mariner mutagenesis of the ciaRH (Martin et al., 2000), endA, comEAC, comFAC, comGAB, and dprA (Bergé et al., 2002), as well as cibABC (Guiral et al., 2005), comM-lytR (Håvarstein et al., 2006) and radC (Attaiech et al., 2008) loci, we concluded that the failure to isolate insertions in a locus occupying a central position on the targeted PCR fragment and the biased distribution observed were strongly indicative of the essentiality of codY, at least in the genetic background and under the plating conditions (CAT-agar) used. [It is of note that a similar conclusion regarding the essentiality of lytR (spr1759) based on the failure to isolate mariner insertions in this locus (Håvarstein et al., 2006) was confirmed in a further study (Johnsborg and Havarstein, 2009).]
Construction of a strain harboring a second (ectopic) copy of codY

The failure to inactivate codY through mariner mutagenesis prompted us to construct a strain harboring a second copy of codY that should tolerate inactivation of one of the two copies. The second copy was inserted at CEP (chromosomal expression platform) (Guiral et al., 2006) under the control of the maltose-inducible promoter, P_M (Fig. 1B). The resulting strain is referred to as codY+/+ hereafter. The duplication of codY had no detectable effect on growth and spontaneous competence induction when cells were grown with 1% maltose, i.e. under conditions leading to full induction of the P_M promoter (Guiral et al., 2006), suggesting that the level of CodY attained with this induction system is not detrimental to the cell (data not shown).

Minitransposon insertions in codY are readily obtained in diploid (codY+/+) cells

A codY+/+ strain (R2349) was then used as recipient for mariner mutagenesis of codY with plasmid pR412 as donor of minitransposon (spc^R cassette; Table 1). In contrast to the failure to inactivate codY using wildtype recipient cells, minitransposon insertions were readily obtained in cells grown in maltose. A codY1-codY2 (Table 1) PCR fragment of which the size was consistent with that predicted for integration of the spc^R minitransposon was observed for 11 out of 20 randomly selected clones. Five of these insertions turned out to inactivate codY as judged from their location (Fig. 1C) and from the inability to introduce them at high frequency in a wildtype recipient (data not shown but see next section).
The isolation of minitransposon insertions in the genuine copy of \textit{codY} suggested that the ectopic copy placed at CEP was able to complement \textit{codY} deficiency. This conclusion was further strengthened by the observation that upon transformation of \textit{codY}^{+/+} cells with a PCR fragment carrying the \textit{codY::spc}^{3A} insertion (generated with the primer pair \textit{codYatg-codYstop}; Fig. 1C and Table 1), \textit{Spc}^R transformants distributed about equally between the genuine \textit{codY} (e.g. strain R2424 the genotype of which is denoted \textit{codY}^{spc3/+}; Fig. 2A) and the ectopic CEP\textsubscript{M}-\textit{codY} loci (e.g. strain R2425 the genotype of which is denoted \textit{codY}^{+/spc3}; Fig. 2B).

\textit{Transformation frequencies of \textit{codY}^{spc3/+} in wildtype and \textit{codY}^{+/+} cells confirm \textit{codY} essentiality}

To unambiguously demonstrate the essentiality of \textit{codY} in our strains, we then used chromosomal DNA from a \textit{codY}^{spc3/+} strain (R2438) as donor in transformation of a wildtype recipient. If \textit{codY} is essential, the survival of transformants harboring the \textit{codY::spc}^{3A} knock-out mutation is predicted to rely on the simultaneous integration of CEP\textsubscript{M}-\textit{codY-kan} (i.e. a double transformation event occurring independently as the two loci are not genetically linked). On the other hand, a \textit{codY}^{+/+} recipient should readily accept the \textit{codY} knock-out. Transformation frequency should therefore be significantly reduced in wildtype cells compared to a \textit{codY}^{+/+} recipient. In full agreement with this prediction, transformation of \textit{codY::spc}^{3A} occurred in \textit{codY}^{+/+} (strain R2350) cells with a frequency close to that of the \textit{str4I} (Str\textsuperscript{R}) reference marker [Note that transformation of the \textit{spc}^R cassette, which requires integration of ~1.1 kb heterologous DNA, is expected to occur with a 2 to 3-fold reduced frequency compared to the point-mutation reference marker \textit{str4I}] (Fig. 3A). In contrast, ~20-
fold reduction in the frequency of Spc\textsuperscript{R} transformants was observed with wildtype (R895) cells (Fig. 3A). The observed frequency was close to that calculated as the product of individual transformation frequencies of Spc\textsuperscript{R} and Kan\textsuperscript{R}. To verify that Spc\textsuperscript{R} transformants obtained with the wildtype recipient had simultaneously acquired resistance to Kan, 10 Spc\textsuperscript{R} transformants were isolated. All of them turned out to carry the CEP\textsubscript{M}-codY-kan construct (data not shown). Altogether, these data demonstrated that codY is an essential gene in S. pneumoniae R800 and its derivatives.

codY is also essential in R6 and in encapsulated D39

R800 was originally derived from R6 through introduction of a suppressor mutation (originating from strain Cl\textsubscript{3}, another D39 derivative) (Tiraby et al., 1975) that greatly improved growth of ami mutants (Lefèvre et al., 1979). This led us to wonder whether the presence of this uncharacterized suppressor was responsible for the essentiality of codY. We therefore introduced the CEP\textsubscript{M}-codY construct in R6 (generating strain R2427) and measured transformation frequencies of codY::spc\textsuperscript{3A} using codY\textsuperscript{spc3/+} chromosomal DNA as donor. Yield of Spc\textsuperscript{R} transformants was significantly lower in R6 than in its codY\textsuperscript{+/+} derivative indicating that codY is essential in this background as well (Fig. 3B).

Inactivation of codY was previously reported in the encapsulated S. pneumoniae D39 strain (Hendriksen et al., 2008). To check whether our negative data could be explained by the use of unencapsulated strains, of different medium and/or plating conditions, we first tried to transform D39 with an amiF1-kan1 PCR fragment carrying codY::spc\textsuperscript{3A} (Fig. 2B), using previously described conditions for plating of codY\textsuperscript{−} transformants on Columbia base agar (Hendriksen et al., 2008). No Spc\textsuperscript{R} transformants (i.e. <10\textsuperscript{-6}) could be obtained (data not
Transformation of codY::spc\textsuperscript{3A} using codY\textsuperscript{spc3A} chromosomal DNA as donor yielded \textasciitilde 20-fold fewer Spc\textsuperscript{R} transformants in D39 than in its codY\textsuperscript{+/+} derivative (strain TD73) indicating that introduction of a codY knock-out in D39 also requires the simultaneous transfer of the complementing ectopic CEP\textsubscript{M}-codY gene (Fig. 3C). In full agreement with this interpretation, Spc\textsuperscript{R} transformants were also Kan\textsuperscript{R}. We concluded that codY is also essential in encapsulated D39 and that this gene must therefore be added to the list of essential pneumococcal genes (Thanassi et al., 2002).

Re-investigation of the previously described codY knock-out reveals the presence of additional suppressor mutations or a chromosomal rearrangement

We then re-examined the previously constructed codY::trim mutant, strain D39\textΔcodY (Hendriksen et al., 2008), as the transformation frequency upon mutant generation was very low (W. Hendriksen, personal communication). PCR analysis of the codY chromosomal region of strain D39\textΔcps\textΔcodY, which was derived from D39\textΔcodY by transformation with a PCR fragment harboring the Δcps::kan cassette, as previously described (Bootsma et al., 2007), confirmed the published structure (data not shown). Preliminary attempts using D39\textΔcps\textΔcodY chromosomal DNA as donor revealed that transformation of the codY::trim construct into R800 derivatives occurred only at very low frequency (data not shown). To facilitate the comparison with transformation experiments reported above, we replaced the codY::trim construct with the codY::spc\textsuperscript{3A} insertion (using as donor a codY1-codY2 PCR fragment) and we introduced the str41 reference marker (using as donor a PCR fragment amplified with the rpsL\textsubscript{3}-rpsL\textsubscript{4} primer pair; Table 1) thus generating strain TD81. Then,
using TD81 chromosomal DNA as donor, we compared transformation frequencies of
\textit{codY::spc}^{3A} in the same pairs of \textit{codY}^{+} and \textit{codY}^{+/+} derivatives of R800, R6 and D39 used in
\textbf{Fig. 3}. Transformation frequencies in \textit{codY}^{+} strains were reduced by 544, 762, and 351 fold,
respectively in R800, R6, and D39, compared to their \textit{codY}^{+/+} isogenic derivatives (\textbf{Fig. 4A-C}). These very large reductions in transformation frequency (compared to the \textasciitilde20-fold
reduction observed when the simultaneous transfer of \textit{codY::spc}^{3A} and CEP\textsubscript{M}-\textit{codY} from a
\textit{codY}^{spc3/C} donor was required; \textbf{Fig. 3}) indicated that transfer of the \textit{codY::spc}^{3A} from strain
TD81 must be accompanied by the transfer of more than one point mutation. Alternatively,
the very low transformation frequency could be accounted for by the requirement for a
simultaneous chromosomal rearrangement. We concluded that the original D39\textsubscript{ΔcpsΔcodY}
strain either contained two additional suppressor mutations that are presumably acting
together to compensate for the absence of CodY and restore cell viability, or harbored a
chromosomal rearrangement allowing survival in the absence of CodY. We tentatively named
it/them \textit{socY} (for suppressor of \textit{codY}).

To get a possible insight into the molecular nature of the mutation(s) involved, we carried
the same transformation experiments but with a pair of \textit{hex} mutant derivatives of R800 (strain
R1818 and its \textit{codY}^{+/+} derivative, R2428). The Hex system of \textit{S. pneumoniae} is known to
correct out some mismatches at the donor-recipient heteroduplex stage in transformation
(\textcite{Claverys 1986}). It is particularly efficient at correcting transition mismatches (i.e.
A/C or G/T) as well as short frameshifts (\textcite{Gasc 1989}). Transformation frequencies with
\textit{hex}^{-} recipient cells were improved \textasciitilde4.5 fold (125 versus 544 fold reduction in the \textit{codY}^{+}
parent compared to the \textit{codY}^{+/+} derivative, in \textit{hex}^{-} and \textit{hex}^{+} strains respectively; \textbf{Fig. 4D}),
which suggests that one of the \textit{socY} mutations is recognized by the Hex system and is
therefore possibly a transition or a short frameshift. Alternatively, the recombination event
leading to the putative chromosomal rearrangement may involve the formation of a heteroduplex intermediate harboring mismatches susceptible to the Hex system.

Effect of codY inactivation on spontaneous competence development

To characterize the role of S. pneumoniae CodY with respect to growth and the regulation of competence, we first tried to deplete CodY making use of a codY^{spc/+} strain, i.e. a strain in which the only functional copy of codY was under the control of the P_M promoter. In light of the failure to obtain a functionally significant depletion of CodY (Supplementary results), we chose to characterize a codY socY strain with respect to spontaneous competence development and growth. Competence was monitored throughout incubation at 37°C by using a transcriptional fusion of the luc gene, which encodes luciferase, to the ssbB gene. The latter is known to be specifically induced at competence. The ssbB::luc fusion thus reports on competence through light emission by luciferase (Prudhomme and Claverys, 2007). We introduced the ssbB::luc transcriptional fusion (using R895 chromosomal DNA as donor) in strains D39Δcps and D39ΔcpsΔcodY socY thus generating strains TD82 and TD83, respectively (Table 1). We then compared competence profiles of strains TD82 and TD83 during growth in C+Y medium with initial pH values between 6.48 and 7.26, since spontaneous competence induction is known to be strongly dependent on the initial pH. For instance, initial pH values between 6.8 and 8.0 affected the timing of occurrence and the level of competence (Chen and Morrison, 1987). While the wildtype parent developed spontaneous competence only in cultures with initial pH values above 7.0 (Fig. 5A-B), the codY mutant could develop competence under acidic conditions, down to an initial pH value of 6.70 (Fig. 5C-D). It is of note that despite the presence of the socY suppressor, codY mutant cells grew...
more slowly than wildtype cells in C+Y medium (Fig. 5A-D). codY mutant cells thus entered the stationary phase of growth after ~270 min incubation compared to ~170 min for wildtype cells. This >50% increase in generation time might indicate that CodY plays an important role in the regulation of pneumococcal growth. Despite the fact that codY mutant cells grew more slowly than wild type, they developed competence at about the same time (e.g. maximum competence after 87 versus 82 min incubation at pH 7.26; Fig. 5A and 5C), which corresponded to OD\textsubscript{492} values of 0.083 and 0.115, respectively. This observation was also consistent with upregulation of competence in the codY mutant. The strong competence-upregulated (cup) phenotype (Martin et al., 2000) displayed by codY mutant cells would be consistent with the hypothesis that CodY normally represses competence under acidic conditions in wildtype cells. However, the uncharacterized suppressor mutations (socY) in strain TD83 could also be responsible for the observed cup phenotype. This prompted us to investigate the phenotype of socY (codY\textsuperscript{+}) cells.

**Impact of socY on spontaneous competence development**

First, to establish whether restoration of codY\textsuperscript{+} would be tolerated in a socY genetic background, strain TD83 was transformed with R2644 chromosomal DNA. R2644 carries the \textit{spc\textsuperscript{90C}} insertion immediately upstream of the codY\textsuperscript{+} gene (see Experimental procedures). Integration of the \textit{spc\textsuperscript{R}} cassette by transformation could thus be accompanied by the removal of the adjacent codY::trim cassette. A failure to survive of Trim\textsuperscript{S} (i.e. codY\textsuperscript{+}) excisants should result in a drastic reduction in the number of Spc\textsuperscript{R} transformants, since recombination events leading to spc integration without the simultaneous deletion of trim are rare due to the reduced distance between the two cassettes (105 bp). The high Spc\textsuperscript{R} to Str\textsuperscript{R} transformant ratio
observed (average value 0.32±0.06) was consistent with the simultaneous occurrence of both
integration (of the spc cassette) and excision (of the trim cassette) events, and suggested that
socY (codY+) cells are viable. To establish this, 4 of 4 randomly chosen Spc\textsuperscript{R} transformants
were first checked to be Trim\textsuperscript{S}. Then, they were shown to readily re-accept codY::trim when
transformed with TD80 chromosomal DNA (Trim\textsuperscript{R}/Str\textsuperscript{R} average ratio of 0.25±0.08 over 15
independent cultures). These data confirmed that the Spc\textsuperscript{R} Trim\textsuperscript{S} transformants were still socY
and indicated that none of the two suppressor mutations or the putative chromosomal
rearrangement is detrimental to pneumococcal cells when CodY is present. One of the Spc\textsuperscript{R}
Trim\textsuperscript{S} clones was retained and named strain TD95. The spc\textsuperscript{90C} cassette was then removed
from strain TD95 by transformation with a PCR fragment generated on R800 chromosomal
DNA with the MP188-MP189 primer pair, followed by phenotypic expression and
segregation in liquid culture (C+Y medium) for 4.5 hours, and plating on CAT-agar without
antibiotic. Individual colony screening was carried out to isolate a Spc\textsuperscript{S} clone, TD96. Upon
transformation of TD96 with TD80 chromosomal DNA, a Trim\textsuperscript{R}/Str\textsuperscript{R} ratio of 0.42±0.07 was
observed indicating that TD96 had remained socY.

The competence profile of TD96 during growth in C+Y medium with initial pH values
between 6.48 and 7.26 was compared with that of TD82 (wild type) and TD83 (codY::trim
socY). TD96 socY (codY+) cells grew more rapidly than parental codY::trim socY cells, but
still more slowly than wild type (entry into stationary phase after ~210 min versus ~170 min;
Fig. 5E-F). Interestingly, socY cells displayed a stronger cup phenotype than codY socY cells
as judged first from their ability to develop spontaneous competence at pH 6.60 (Fig. 5F) and
second, from the very early development of competence (36 min at pH 7.26) compared to
both wildtype and codY socY cells (Fig. 5). Thus, the socY mutations alone confer a strong
cup phenotype. The net effect of codY inactivation in this background is to attenuate this cup
phenotype. From these data, it is therefore difficult to conclude that CodY acts as a repressor of competence in pneumococcal cells.

Whole genome sequence comparison suggests fatC and amiC mutations suppress inviability of codY mutants

In an attempt to identify the socY (suppressor) mutations, whole genome resequencing of strains D39 and D39ΔcodY was performed (Experimental procedures). Compared to the previously published D39 NCTC 7466 genome (Lanie et al., 2007), the D39ΔcodY strain had 14 mutations also found in its parent. As our D39 was obtained originally from NCTC (via T. Mitchell), these mutations presumably have arisen during laboratory cultivation. None of them appeared to alter an important function (Table S1), which is consistent with the fact that this D39 strain displayed full virulence in a mouse model (Hendriksen et al., 2008).

The codY mutant had also mutations flanking the trim cassette [a C→T transition, a GT→AGC frameshift and a T→A transversion, respectively at positions -175, +681/682 and +724 (positions are given with respect to the ATG of codY)]. All these changes occurred in between the trim cassette and the oligonucleotide primers used to amplify the codY region (Hendriksen et al., 2008), and were presumably introduced during polymerase chain reaction. The transition mutation is in itself sufficient to lower the frequency of integration of the codY::trim cassette by ~5-fold during transformation of mismatch-repair proficient strains.

Most relevant with respect to the question of CodY essentiality, the codY mutant had, in addition, a mutation in fatC and a second, variable mutation in amiC (Fig. 6A). The former mutation (a C→T transition), fatC<sup>C496T</sup> changed a CAA (Gln) codon into TAA (stop) in a gene belonging to the fatD-fatC-fecE-fatB operon ( spd_1649-1652 in D39; spr1684-1687 in R6;
sp1869-1872 in TIGR4) (Fig. 6A, top). This operon [also called piuBCDA or pitl (Brown et al., 2002)] encodes the major ferric iron/heme [fatB has been shown to bind hemin (Tai et al., 2003)] transporter of *S. pneumoniae* (Ulijasz et al., 2004). The fatC<sup>G496T</sup> change is predicted to result in the synthesis of a truncated FatC protein (165 instead of 318 aa) and was detected in 100% of forward and reverse reads. As concerns the base variations in *amiC*, which encodes a 598-aa protein and belongs to the *amiACDEF* operon (*spd*_1671-1667 in D39; *spr1707*_1703 in R6; *sp1891*_1887 in TIGR4), three types of mutations were observed: two single-base changes (#2 and #3 in Fig. 6A, bottom) and a more complex mutation (#1 in Fig. 6A, bottom). These variations were detected in only a fraction of sequence runs. Mutations #2 (*amiC*<sup>G1438T</sup>) and #3 (*amiC*<sup>G1459T</sup>) change a GAC (Asp) codon into TAC (Tyr) (aa 480 and 487, respectively); these mutations were detected in 30.8% of forward and 11.1% of reverse reads (total with variation: 19%), and in 57.1% of forward and 40.9% of reverse reads (total with variation: 47%), respectively. Interestingly, both changes affect an Asp aa conserved in the entire family of Opp (oligopeptide), Dpp (dipeptide) and App (nickel) permeases (our observations), which suggests that they are functionally important and that the corresponding AmiC proteins may have lost their activity. The complex mutation #1 consisted of a G→TT frameshift (*amiC*<sup>G246TT</sup>) and the almost adjacent A→C change (*amiC*<sup>A248C</sup>); both mutations shared the same sequencing parameters (detection in 25.0% of forward and 33.3% of reverse reads), which strongly suggests that they resulted from a single mutational event (total with variation, 29%). This mutation could thus be described as a GGA→TTGC change. [In fact, this change had already been observed during analysis of mismatch repair specificity in *S. pneumoniae* and referred to as *amiA29* (at this time, the *ami* locus was thought to be a single gene, hence the name *amiA*) (Gasc et al., 1989) (for a molecular explanation of this mutational event, see Fig. S2).] This complex mutation is predicted to result in the synthesis
of a truncated protein harboring the first 81 aa of AmiC fused to 13 ‘new’ aa (resulting from the frameshift).

At first sight, the presence of changes #1, #2 and #3 in only a fraction of sequence runs appeared puzzling and suggested their late occurrence in an original ΔcodY fatC^{C496T} clone.

As a first attempt to establish whether an ami mutation was required for tolerance of codY, we analyzed the ami locus of D39ΔcpsΔcodY, considering it a subclone of the original D39ΔcodY strain since it was generated by transformation of this strain with a PCR fragment harboring the Δcps::kan cassette. Taking advantage of the fact that amiC-F mutations normally confer resistance to methotrexate (Mtx^{R}), transformation of an Mtx^{S} strain (R1501) with a series of PCR fragments covering the ami region of strain D39ΔcpsΔcodY was used to localize any ami mutation (Fig. S3). Transformation data were consistent with the presence of mutation #2 or #3 in strain D39ΔcpsΔcodY; DNA sequencing confirmed that this strain had inherited the latter mutation (amiC^{G1459T}). These data prompted us to directly confirm the mixed structure population with respect to amiC of the original D39ΔcodY strain. An aliquot of the original stock was plated, 16 individual clones were picked and amiC (and fatC) regions were sequenced (Table S2). All clones turned out to harbor one of the three amiC mutations identified during whole genome sequencing (and 11 out of 11 clones sequenced carried the fatC mutation). Interestingly, the overall distribution between the three types was in very good agreement with that inferred from the analysis of genome sequence data (Table S2). It is also of note that 3 clones harbored change #1 (i.e. both amiC^{G246TT} and amiC^{A248}), which provided support to the view that a single molecular event accounts for a complex change (Fig. S2).

Altogether, these results strongly suggested that inactivation of amiC was required for tolerance of codY inactivation. We tentatively concluded from these observations that the combination of fatC and amiC mutations suppresses inviability of codY mutants.
The fatC amiC combination allows tolerance of codY inactivation

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<sup>C355T</sup>) [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<sup>23C</sup> cassette and/or the amiC9 mutation (Fig. 6A) to accept codY inactivation.

Strain (R246) and its amiC9 (R3003), fatC::cat<sup>23C</sup> (R3002) and amiC9 fatC::cat<sup>23C</sup> (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<sup>-</sup> 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<sup>G1459T</sup> and fatC<sup>C496T</sup> 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<sup>23C</sup> 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.
Growth and spontaneous competence development of wild type (TD82) and amiC9 (TD84), fatC::cat^{23C} (TD138), amiC9 fatC::cat^{23C} (TD135) and amiC9 fatC::cat^{23C} codY::trim (TD154) mutant strains were then compared (Fig. S5). Both fatC and amiC mutant strains displayed a cup phenotype, consistent in the latter case with the phenotype of obl mutants (Alloing et al., 1998). The cup phenotype of the double mutant was most similar to that observed with the amiC mutant (Fig. S5) and with the socY strain (Fig. 5E-F). Finally, while codY inactivation resulted in slower growth confirming the observation in Fig. 5, it did not significantly attenuate the cup phenotype observed with the amiC fatC double mutant (compare TD135 and TD154, Fig. S5).

Discussion

CodY is a pleiotropic regulator in low-GC Gram-positive bacteria involved in the control of different processes like aa uptake, competence, sporulation and virulence. We first established that codY is an essential gene in the D39 strain of S. pneumoniae and in several of its laboratory derivatives by using different genetic approaches (mariner mutagenesis, complementation, transfer of mutations in different genetic backgrounds; Fig. 1-3). We then showed that a previously obtained codY mutant (D39ΔcodY) used to identify the CodY regulon (Hendriksen et al., 2008) contains additional suppressor mutations, called socY, one of which was concluded to correspond to a transition mismatch or a frameshift (Fig. 4). We also showed that both the codY socY and socY strains displayed a competence upregulated or cup phenotype (Fig. 5).

Whole genome sequencing of strains D39 and D39ΔcodY undertaken to identify the socY suppressors revealed the presence of mutations in fatC and amiC, which encode the
membrane permease component of ferric iron/heme and oligopeptide ABC transporters, respectively (Fig. 6A). This combination of mutations was unambiguously demonstrated to be required to allow tolerance of codY inactivation (Fig. 6B). These results fully confirm the conclusions from codY transfer experiments, including the presence of a mutation susceptible to mismatch repair (Fig. 4D), i.e. the fatC<sup>C496T</sup> transition.

Proposed scenario for the initial stabilization/survival of D39ΔcodY cells

Genome sequence clearly indicated that the fatC mutation arose first. However, genome sequence data also strongly suggested that inactivation of amiC is absolutely required for tolerance of codY inactivation. The finding that three different amiC mutations occurred in the D39ΔcodY strain, as confirmed by individual subclone analysis (Table S2), implied a strong selection pressure (growth advantage) for amiC mutant derivatives. The amiC mutations presumably arose independently in an otherwise codY fatC lineage.

It is of note that the D39ΔcodY strain described in Hendriksen et al. (2008) was obtained by backcrossing D39 using chromosomal DNA isolated from an initial D39ΔcodY::trim transformant. Despite this careful strategy, D39ΔcodY::trim turned out to contain two additional mutations, as shown in this study. While a second round of transformation normally eliminates unlinked mutations, it is obviously unable do so when the unlinked mutations are absolutely required for survival. In addition, we noticed that the two suppressor mutations are not very distant. The two closest mutations (fatC<sup>C496T</sup> and amiC<sup>G1459T</sup>), located 17,987 nt apart, could frequently be carried by the same DNA fragment. Careful comparison of transformation frequencies with a well-characterized reference marker (preferably present
on the same chromosomal DNA) is thus the only way to establish unambiguously that any construct is well tolerated and does not affect cell viability.

 CodY, socY and competence

It was implicit in our working hypothesis connecting competence regulation to oligopeptide uptake via a global regulator (like CodY) sensing aa pools (see Introduction) that this regulator would act as a repressor of competence (Claverys et al., 2000). Our data rather suggest that, in contrast to this expectation, inactivation of codY has no major effect on spontaneous competence, most of the effects seen being attributable to the socY mutations (Fig. 5 and Fig. S5). In fact, the amiC mutation is in itself sufficient to account for the strong derepression of competence development observed in D39ΔcodY cells (Fig. S5). It is of note that despite the cup phenotype documented in this study for the D39ΔcpsΔcodY strain (Fig. 5), previous transcriptome analysis did not reveal induction of any com gene (Hendriksen et al., 2008); presumably, culture conditions used for mRNA extraction did not lead to differential induction of the com regulon between the codY mutant and its parent, competence being either similarly induced or repressed for both strains. While normal Ami functioning somehow leads to competence repression, CodY is either neutral (Fig. S5) or possibly acts as an activator of competence, the latter conclusion being suggested by the attenuation of the cup phenotype of amiC fatC cells upon introduction of the codY knock-out (Fig. 5). If confirmed, the role of S. pneumoniae CodY with respect to competence regulation would thus differ from that of its B. subtilis orthologue, which represses competence (Serror and Sonenshein, 1996), suggesting that the impact of nutrient deprivation on competence induction is opposite in B. subtilis and S. pneumoniae or at least that nutritional signals are conveyed in a very different way in these
two species. However, since the impact of codY inactivation on competence could so far be evaluated only in complex genetic backgrounds (i.e. mutant for both amiC and fatC), further work using different approaches, such as a transient depletion of CodY, would be necessary to evaluate the exact role of CodY in the regulation of pneumococcal competence.

CodY, socY and previous transcriptome studies

The finding that the D39ΔcodY strain used in a previous study (Hendriksen et al., 2008) was in fact a codY amiC fatC triple mutant raises the question of the respective contribution of each mutation to the phenotypes previously attributed solely to the inactivation of codY. As concerns transcriptome data, upregulation of aliA (aliB was also upregulated but only 1.8-fold), amiA-amiC-amiD and fatD-fatC-fecE-fatB was reported in ΔcodY (Hendriksen et al., 2008). Binding of CodY to P_amiA (as well as to P_aliB) was consistent with a direct regulation of oligopeptide uptake by CodY. CodY thus exhibited a strong affinity for P_amiA although the effect of BCAA addition was limited (1.56-fold versus 2 to 20-fold enhancement for other promoters). On the other hand, the Kds (the CodY concentration at which 50% of the fragment is shifted) for the P_fatD promoter was higher than the highest concentration tested (2,000 nM) indicative of a rather weak affinity and was unaffected by addition of BCAs, contrary to most other CodY-regulated promoters (Hendriksen et al., 2008). Possibly, the binding of CodY to P_fatD is affected in the presence of other regulators (see below), as fat regulation is clearly multifactorial (Ulijasz et al., 2009). Alternatively, since ΔcodY cells used to prepare mRNA for transcriptome analysis were also mutant for fatC (i.e. they lacked the main iron transporter of S. pneumoniae), the upregulation of the fatD-fatC-fecE-fatB operon could be a reflection of (inefficient) attempts at iron homeostasis by increasing expression of
this transporter rather than the consequence of a lack of repression in the absence of CodY. The same reasoning may apply to dpr gene expression. This gene (spd_1402 in D39; spr1430 in R6; sp1572 in TIGR4) encodes a conserved iron storage-peroxide resistance protein (Ulijasz et al., 2004) that is possibly essential (Pericone et al., 2003). It was found to be downregulated in the codY mutant (Hendriksen et al., 2008). Depletion of iron in codY mutant cells resulting from fatC inactivation could possibly account for the observed reduction in dpr expression without implying any direct regulation by CodY. This would be consistent with the failure to identify a sequence resembling the CodY box upstream of the dpr gene. Clearly, additional work is necessary to establish whether CodY regulates dpr expression directly or indirectly.

CodY, socY and previous virulence studies

Similarly, the presence of amiC and fatC mutations in the D39ΔcodY strain used in a previous study (Hendriksen et al., 2008) raises the question of their respective contribution to the virulence phenotypes previously attributed solely to codY inactivation. The importance of iron for bacterial growth and virulence is well established. Thus, signature-tagged mutagenesis (STM) screens for genes essential during pneumococcal pneumonia identified insertions in genes belonging to each of the three iron transporters characterized in S. pneumoniae (Brown et al., 2001; Brown et al., 2002), piuB (i.e. fatD), pitB (pitADBC operon) and piaA (piaBCD operon) (Hava and Camilli, 2002). These iron uptake systems have been demonstrated to be important for full pneumococcal virulence, especially upon simultaneous mutation of two operons (Brown et al., 2001; Brown et al., 2002). Single mutation of piuB resulted in only a
mild reduction of virulence in a pneumonia model, while no attenuated phenotype was observed in systemic infection (Brown et al., 2001).

Several studies have indicated a role for the Ami-AliA/AliB permease, encoded by the amiACDEF operon and the aliA and aliB genes, in pneumococcal virulence. Mutants in amiA and amiC displayed diminished adherence to pulmonary epithelial cells in vitro (Cundell et al., 1995), and aliB and amiACD were identified in STM screens of pneumococcal pneumonia in a serotype 3 and 4 background, respectively (Lau et al., 2001; Hava and Camilli, 2002). Furthermore, using a collection of aliA, aliB and amiA single or triple mutants, the Ami-AliA/AliB permease was shown to be required for successful nasopharyngeal colonization, but not for pneumococcal pneumonia, with the most pronounced phenotype for the triple and the aliA and amiA single mutants (Kerr et al., 2004).

Inactivation of codY was previously reported to result in reduced adherence to nasopharyngeal cells and reduced colonization in a mouse model of pneumococcal infection (Hendriksen et al., 2008). However, no significant differences in bacterial loads between wild type and the codY mutant were observed in pneumonia and bacteremia models of infection. In light of the above, we consider it unlikely that the truncation of fatC contributed to the virulence phenotype associated with codY inactivation. On the other hand, mutation of amiC may, at least partially, have been responsible for the observed attenuation during colonization, but further experiments are needed to exactly determine the impact of the amiC mutation on the virulence phenotype of the codY mutant.

Why is CodY essential in S. pneumoniae?
The finding that CodY is essential suggests that this regulator controls genes/functions that are crucial for *S. pneumoniae*. The demonstration that mutations in *fatC* and *amiC* fully restore viability of *codY* mutant cells may provide some clue as to these functions. As the genome sequence analysis of D39Δ*codY* strongly suggested that the *fatC* mutation arose first during establishment of the *codY* mutant construct, it is tempting to speculate about a possible toxicity of iron in cells lacking CodY. If CodY is a repressor of the *fatD-fatC-fecE-fatB* operon as previously concluded (Hendriksen et al., 2008), derepression of iron uptake in its absence may lead to accumulation of toxic concentrations of iron within the cell, thus creating a strong selection pressure for the emergence of *fat* mutants. In this context, what could account for the concomitant accumulation of *amiC* mutations? Considering only Ami and CodY, it could be speculated that *codY* inactivation leading to derepression of the many transporters of aa controlled by CodY and of Ami results in lethal imbalance of aminoacid pools. However, this explanation is not readily connected to the co-occurrence of *fatC* inactivation. In search of a possible direct connection, we came across the observation that the housekeeping dipeptide permease of *Escherichia coli* allows utilization of heme as an iron source (Letoffe et al., 2006). Heme utilization has thus been shown to require a permease made up of DppBCDF (dipeptide inner membrane transporter) as the ABC transporter and either MppA (periplasmic L-alanyl-γ-D-glutamyl-meso-diaminopimelate binding protein) or DppA (periplasmic dipeptide binding protein) as the substrate-binding protein. As *S. pneumoniae* is devoid of a Dpp system, it is tempting to speculate that the Ami-Obl oligopeptide transporter homologous to Dpp could allow heme utilization as an iron source. The simultaneous involvement of CodY in *ami-obl* and *fat-fec* repression, and in *dpr* activation would, upon *codY* inactivation, generate a severe oxidative stress because of the simultaneous derepression of iron uptake via two transporters and depletion of the iron storage-peroxide resistance Dpr protein (Fig. 7), hence the observed accumulation of *fat* and
ami mutations in the ΔcodY background. It may not be a mere coincidence that the ami and fat mutations which restore viability of codY mutant cells lead to competence (X-state) derepression (Fig. 5 and Fig. S5). Since X-state is considered a pneumococcal substitute for SOS (Claverys et al., 2006; Prudhomme et al., 2006), its induction may counteract oxidative stress in codY mutant cells. Further investigation should reveal whether induction of the X-state is required for tolerance of codY inactivation.

Transcriptome analysis revealed a striking parallel between CodY and RitR, an orphan two-component signal transduction response regulator. Increased transcription of piuB and piuA (i.e. fatD and fatB), as well as of amiC and decreased expression of dpr were reported in ritR mutant cells (Ulijasz et al., 2004) suggesting that CodY and RitR share these targets (Fig. 7). RitR was shown to bind three sites (RRB1-3; Fig. 6A) in the promoter region of the fat operon (Ulijasz et al., 2004). Recently, regulation of RitR binding at the fat promoter by a Ser-Thr kinase-phosphatase, StkP-PhpP, was documented (Ulijasz et al., 2009). While RitR and PhpP jointly participate in complex formation at the fat promoter in vitro, addition of StkP was shown to disrupt the complexes (Ulijasz et al., 2009). This finding was consistent with DNA microarray analyses of transcripts from an stkP knock-out showing dependence of fat expression on StkP (Saskova et al., 2007). The overlap between the CodY box (AATTGTCAGAAATT located 3 nucleotides upstream of the -35 promoter box) and the first RitR box (Fig. 6A) suggests that CodY may also interfere with RitR binding and adds a degree to the complexity of fat regulation. CodY may thus represent one of the additional fat regulators, the existence of which was proposed to account for the failure to detect the opposite effects predicted for the individual deletion of phpP and stkP (Ulijasz et al., 2009).

In line with the oxidative stress hypothesis (Fig. 7), ritR mutant cells were shown to display greatly increased susceptibility to streptonigrin, which requires the presence of intracellular iron, as well as to hydrogen peroxide (Ulijasz et al., 2004). Iron overload due to derepression
of fat could be responsible for the latter by increasing intracellular free iron concentration and therefore the potential for synthesis of reactive oxygen intermediates. If both CodY and RitR are important for iron homeostasis and the reason for CodY essentiality is to prevent oxidative stress, what about the viability of ritR mutant cells? As there was no mention of difficulty in generating and/or growing ritR mutants, CodY might be more important for ami, fat and dpr regulation than RitR. Alternatively, RitR and CodY could be equally important and the problematic viability of ritR mutant cells has been overlooked. It would therefore be interesting to check whether a previously constructed ritR mutant is readily transferred by transformation.

Concluding remarks

Further work with other clinical isolates of S. pneumoniae is necessary to establish whether the essentiality of CodY is a general feature of this species. To the best of our knowledge, this is the first report in any bacterium that a member of the CodY family is essential. Is this situation unique to S. pneumoniae? It would be interesting to investigate whether CodY is essential in species closely related to S. pneumoniae and in streptococci in general. It is possible that CodY is also essential in other species but that the presence of suppressor mutation(s) has been overlooked. Our observations may thus prompt careful reexamination of the viability of the codY mutants previously constructed in other species. More generally, the above described tests of acceptance frequencies by transformation should be used routinely when working with ‘important’ genes, to prevent the presence of suppressors going undetected in the future. In any case, because of its essentiality in a major human pathogen, CodY constitutes a potentially interesting new therapeutic target.
Experimental procedures

Bacterial strains, culture and transformation conditions

*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
challenge with a 10 ml overlay containing chloramphenicol (4.5 µg ml\(^{-1}\)), erythromycin (0.05 µg ml\(^{-1}\)), kanamycin (250 µg ml\(^{-1}\)), methotrexate (2.2 µg ml\(^{-1}\)), spectinomycin (100 µg ml\(^{-1}\)), streptomycin (200 µg ml\(^{-1}\)) or trimethoprim (20 µg ml\(^{-1}\)), after phenotypic expression for 120 min at 37°C.

Mutagenesis and duplication of codY

Insertions of \textit{kan} (\textit{Kan}\textsuperscript{R}) or \textit{spc} (\textit{Spc}\textsuperscript{R}) minitransposons were generated by \textit{in vitro mariner} mutagenesis as described (Prudhomme \textit{et al.}, 2007). Plasmids used as a source for the minitransposons were pR410 and pR412, respectively (Table 1). Briefly, plasmid DNA (~1 µg) was incubated with a target PCR fragment (indicated in the legend of Fig. 1) in the presence of purified Himar1 transposase, leading to random insertion of the minitransposon within the fragment. Gaps in transposition products were repaired as described (Prudhomme \textit{et al.}, 2007) and the resulting \textit{in vitro}-generated transposon insertion library was used to transform \textit{S. pneumoniae}. Location and orientation of minitransposon insertions were determined as previously described (Prudhomme \textit{et al.}, 2007) through PCR reactions using primers MP127 or MP128 in combination with either one of the primers used to generate \textit{codY} PCR fragments (Table 1). Cassette-chromosome junctions were sequenced for some insertions as indicated in the legend of Fig. 1.

Placement of a second copy of \textit{codY} under the control of the maltose-inducible \(P_M\) promoter at CEP was achieved by cloning into \textit{NcoI-BamHI} digested pCEP2 plasmid DNA a \textit{codY} PCR fragment generated using the codYatg-codYstop primer pair (Table 1) and digested with \textit{BamHI} and \textit{NdeI}. The resulting recombinant plasmid pCEP-codY was used as donor in transformation of strain R1501 followed by selection for a \textit{Kan}\textsuperscript{R} transformant, thus generating
strain R2349 (Table 1). Plasmid pCEP2 was generated in this study as a high copy number derivative of plasmid pCEP (Guiral et al., 2006). Briefly, an EcoRI-PstI fragment from pCEP was ligated to EcoRI-PstI digested pKL147 (Table 1) to replace the pSC101 replication machinery of pCEP by the pBR replication machinery and Ap<sup>R</sup> resistance gene of pKL147.

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

To replace codY::trim or codY::spc insertions by codY<sup>+</sup>, we took advantage of the kan<sup>90C</sup> mariner insertion. This insertion is located immediately upstream the CodY binding site (CYB) in the codY promoter region (Fig. 1A) and does not inactivate codY; we used it as a marker to select for the re-introduction of the codY<sup>+</sup> gene by co-transformation with Kan<sup>R</sup>. To allow the use of a similar strategy for replacement of codY::trim in kan<sup>R</sup> strains (such as TD83), the kan<sup>90C</sup> cassette was exchanged with the spc<sup>R</sup> cassette by transformation of strain R2641 with plasmid pR412 DNA, selecting for Spc<sup>R</sup> transformants to generate strain R2644 (genotype referred to as spc<sup>90C::codY<sup>+</sup></sup>; Table 1). The exchange is based on the presence of DNA homology at the borders of the synthetic spc and kan minitransposons, allowing exchange of the resistance cassette genes by homologous recombination during transformation.

**Whole genome sequencing of D39 and D39ΔcodY**

Roche 454 FLX whole genome sequencing was performed by Agowa Genomics (Berlin, Germany) using genomic DNA isolated from mid-log cultures by the Genomic DNA kit.
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For each strain, a shotgun library and a 3-kb span paired end library were generated according to Roche standard protocols, mixed in equal parts (about 400,000 beads from each library) and sequenced using default settings on a ¼ picotiterplate. A total of 240496 reads of which 65336 contained paired ends were obtained for D39ΔcodY (29-fold coverage), and 210631 reads with 60676 paired ends were obtained for D39 (25-fold coverage). De novo assembly was carried out using the Roche 454 Newbler software (Release 2.3 (091027_1459), resulting in 68 contigs in 5 scaffolds for D39ΔcodY, and 85 contigs in 5 scaffolds for D39.

Data from the sequencing runs were mapped to the reference D39 strain (Acc.no.: NC_008533.1) and the variations thereto scored using the Roche 454 Reference Mapper software [Release 2.3 (091027_1459)].

Sequencing of amiC and fatC of individual D39ΔcodY clones

The amiC and fatC sequence of the D39ΔcodY population was verified by a PCR-sequencing approach. To this end, chromosomal DNA was isolated from individual clones by cetyltrimethylammonium bromide (CTAB) extraction as described previously (van Soolingen et al., 1994). The amiC and fatC loci were PCR-amplified under standard conditions using, respectively, primers HBDamiCF1 and HBDamiCR2 and HBDfatCF and HBDfatCR (Table 1). Subsequently, both strands were sequenced using the same primers used for PCR as well as internal primers HBDamiCR1 and HBDamiCF2 (Table 1) in case of amiC.
Acknowledgements

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Supplementary material

The following Supplementary Material is available for this article:

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

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

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

**Fig. S3.** Localization of $ami$ mutation in strain D39ΔcpsΔcodY via transformation.

**Table S2.** The original D39ΔcodY strain displays a mixed population structure with respect to $amiC$.

**Fig. S4.** Distribution of spc mariner minitransposon insertions in the fat-fec operon.
Fig. S5. Effect of ami, fat and ami fat inactivation on growth and spontaneous competence development.

References


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

<table>
<thead>
<tr>
<th>Strains</th>
<th>Genotype/description</th>
<th>Source/reference</th>
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<tbody>
<tr>
<td><strong>Streptococcus pneumoniae</strong></td>
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<td>D39</td>
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<td>NCTC 7466</td>
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<td>(Hendriksen et al., 2008)</td>
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<td>D39 Δcps::kan; KanR</td>
<td>(Hendriksen et al., 2008)</td>
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<tr>
<td>D39ΔcpsΔcodY</td>
<td>D39 Δcps::kan ΔcodY::trim (socY)c; KanR, TrimR, (MtxR)c</td>
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<td>Unencapsulated derivative of D39</td>
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<td>R6 derivative</td>
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<td>This study</td>
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R2425 | R2350 but ectopic codY inactivated by mariner insertion  
This study

| spec<sup>3A</sup> (the resulting codY combination is denoted codY<sup>+/spc</sup>);
| Kan<sup>R</sup>, Cm<sup>R</sup>, Spc<sup>R</sup>

R2427 | R6 but codY<sup>+</sup>/; Kan<sup>R</sup>
| This study

R2428 | R1818 but codY<sup>+</sup>/; Ery<sup>R</sup>, Kan<sup>R</sup>
| This study

R2430 | R895 but pmalR (pAPM22); Cm<sup>R</sup>, Ery<sup>R</sup>
| This study

R2432 | R2424 but pmalR (pAPM22); Cm<sup>R</sup>, Ery<sup>R</sup>, Kan<sup>R</sup>, Spc<sup>R</sup>
| This study

R2437 | R2349 but str41; Kan<sup>R</sup>, Str<sup>R</sup>
| This study

R2438 | R2437 but codY<sup>spc3</sup>; Kan<sup>R</sup>, Spc<sup>R</sup>, Str<sup>R</sup>
| This study

R2549 | R895 but amiC9; Cm<sup>R</sup>, Mtx<sup>R</sup>
| This study

R2641 | R895 but kan<sup>90C::codY</sup>, str41; Cm<sup>R</sup>, Spc<sup>R</sup>, Str<sup>R</sup>
| This study

R2644 | R2641 but spc<sup>90C::codY</sup>; Cm<sup>R</sup>, Spc<sup>R</sup>, Str<sup>R</sup>
| This study

R2737 | R895 but CEP<sub>M</sub>; Cm<sup>R</sup>, Kan<sup>R</sup>
| This study

R3002 | R246 but fatC::cat<sup>23C</sup> (from TD131); Cm<sup>R</sup>, Ery<sup>R</sup>
| This study

R3003 | R246 but amiC9; Ery<sup>R</sup>, Mtx<sup>R</sup>
| This study

R3004 | R3002 but amiC9; Cm<sup>R</sup>, Ery<sup>R</sup>, Mtx<sup>R</sup>
| This study

TD73 | D39 but codY<sup>+</sup>/; Kan<sup>R</sup>
| This study

TD80 | D39∆cps∆codY (socY)<sub>]</sub> but str41; Kan<sup>R</sup>, Str<sup>R</sup>, Trim<sup>R</sup>
| This study

TD81 | TD80 but codY::spc<sup>3A</sup>; Kan<sup>R</sup>, Spc<sup>R</sup>, Str<sup>R</sup>
| This study

TD82 | D39∆cps but ssbB::luc (ssbB<sup>+</sup>); Cm<sup>R</sup>, Kan<sup>R</sup>
| This study

TD83 | D39∆cps∆codY but ssbB::luc (ssbB<sup>+</sup>); Cm<sup>R</sup>, Kan<sup>R</sup>, Trim<sup>R</sup>
| This study

TD84 | TD80 but amiC9; Cm<sup>R</sup>, Kan<sup>R</sup>, Mtx<sup>R</sup>
| This study

TD95 | TD83 but spc<sup>90C::codY</sup>; Cm<sup>R</sup>, Kan<sup>R</sup>, Spc<sup>R</sup>
| This study

TD96 | TD95 but codY<sup>+</sup>; Cm<sup>R</sup>, Kan<sup>R</sup>
| This study

TD129 | D39∆cps but fatC::spc<sup>23C</sup>; Kan<sup>R</sup>, Spc<sup>R</sup>
| This study

TD130 | TD129 but amiC9; Kan<sup>R</sup>, Mtx<sup>R</sup>, Spc<sup>R</sup>
| This study

TD131 | TD129 but fatC::cat<sup>23C</sup>; Cm<sup>R</sup>, Kan<sup>R</sup>
| This study

TD135 | TD130 but ssbB::luc (ssbB<sup>+</sup>); Cm<sup>R</sup>, Kan<sup>R</sup>, Mtx<sup>R</sup>, Spc<sup>R</sup>
| This study
| TD138 | TD129 but \textit{ssbB::luc (ssbB*)}; \textit{CmR, KanR, SpcR} | This study |
| TD154 | TD135 but \textit{ΔcodY::trim}; \textit{CmR, KanR, MtxR, SpcR, TrimR} | This study |

### Plasmids

<table>
<thead>
<tr>
<th>plasmid</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pAPM22</td>
<td>pLS1 derivative carrying the \textit{malR} gene; Ery\textsuperscript{R}</td>
<td>(Puyet \textit{et al.}, 1993)</td>
</tr>
<tr>
<td>pCEP</td>
<td>pSC101 derivative (i.e. low copy number plasmid) carrying CEP; Spc\textsuperscript{R}, Kan\textsuperscript{R}</td>
<td>(Guiral \textit{et al.}, 2006)</td>
</tr>
<tr>
<td>pCEP2</td>
<td>pKL147 derivative (i.e. high copy number plasmid) carrying an \textit{EcoRI/PstI} fragment from pCEP; Spc\textsuperscript{R}, Kan\textsuperscript{R}, Ap\textsuperscript{R}</td>
<td>This study</td>
</tr>
<tr>
<td>pCEP2-\textit{codY}</td>
<td>pCEP2 derivative carrying \textit{codY} under \textit{P\textsubscript{M}} control; Spc\textsuperscript{R}, This study Kan\textsuperscript{R}, Ap\textsuperscript{R}</td>
<td>This study</td>
</tr>
<tr>
<td>pEM\textit{cat}</td>
<td>ColE1 derivative carrying a \textit{CmR} \textit{mariner} minitransposon;</td>
<td>(Akerley \textit{et al.}, 1998) Ap\textsuperscript{R}, Cm\textsuperscript{R}</td>
</tr>
<tr>
<td>pKL147</td>
<td>pUS19 derivative containing \textit{gfpmut2} fused to the 3\textsuperscript{'} end of \textit{dnaX} with a linker; Spc\textsuperscript{R}, Ap\textsuperscript{R}</td>
<td>(Lemon and Grossman, 1998)</td>
</tr>
<tr>
<td>pR410</td>
<td>pEM\textit{cat} derivative carrying a \textit{KanR} (\textit{kan} gene) \textit{mariner} (Sung \textit{et al.}, 2001) minitransposon; Ap\textsuperscript{R}, Kan\textsuperscript{R}</td>
<td>This study</td>
</tr>
<tr>
<td>pR412</td>
<td>pEM\textit{cat} derivative carrying a \textit{SpcR} (\textit{aad9} gene, also called \textit{spc} \textit{mariner} minitransposon; Ap\textsuperscript{R}, Spc\textsuperscript{R}</td>
<td>(Martin \textit{et al.}, 2000)</td>
</tr>
</tbody>
</table>

### Primers

<table>
<thead>
<tr>
<th>\textit{Primer}</th>
<th>\textit{Sequence}\textsuperscript{d}; \textit{gene}; \textit{position}\textsuperscript{g}</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ami1</td>
<td>GCGCAACACGGCTCTAAGGG; \textit{amiA}; +1815</td>
<td>This study</td>
</tr>
<tr>
<td>ami2</td>
<td>TCAGGAATTCTGCTGCATTAT; \textit{amiC}; +1257</td>
<td>This study</td>
</tr>
<tr>
<td>ami4</td>
<td>CCTGACTCACCTACCAAGGCTA; \textit{amiD}; +712</td>
<td>This study</td>
</tr>
<tr>
<td>ami5</td>
<td>CCTTCACCGAAGGAAATTTCTA; \textit{amiE}; +121</td>
<td>This study</td>
</tr>
<tr>
<td>ami6</td>
<td>TTAGCTGACTTTCAACCCACTACA; \textit{amiF}; +1027</td>
<td>This study</td>
</tr>
<tr>
<td>amiF1</td>
<td>GCCTTGCTTTTCACGGGTACCAAT; \textit{amiF}; +789</td>
<td>This study</td>
</tr>
</tbody>
</table>
AM40       AGAGTTTCGGATGGTTTGGA; treR; +347  This study

codY1      CAAGGATCAGTTTTCCCATATTTTCG; codY; +1636 This study

codY2      CTTCGTGTCCTTCGTGACTTTTA; codY; -1004 This study

codYatg    tgaatcATGaCACATTTATTAGAAAAACTAG; codY; 0  This study

codYstop   aaatgtagtatTTTGTCATTAGAATCTCTTTTC; codY; +797 This study

fat1       GCGAACAATGATTTTACTGG; fatD; -659 This study

fat2       TCTCACCAGTCTTTCCACCC; fatB; +1388 This study

fatC1      TAAAAAGCACATACCAAGC; fatC; -9  This study

fatC2      TAAAAAGAATAAGAAGCCACCC; fatC; +909 This study

HBDamiCF1  ACGGCTGATAAAACGTGATAA; amiC; +145  This study

HBDamiCF2  GTCGTTGGTCTTGTCTTCAT; amiC; +1381 This study

HBDamiCR1  TAAAATCTCCAAAAGTCCAA; amiC; +343 This study

HBDamiCR2  CGCATCAATAGTTTCAGAGG; amiC; +1568 This study

HBDfatCF   ACACGTGATAAGCAAGACCT; fatC; +376 This study

HBDfatCR   CAATATCTGAGCCGTTTCTC; fatC; +645 This study

kan1       ATCATGTCCCTTTTCCCCGTCCAC; kan; +191 This study

MP127      CCGGGGACTTTATCAGCCAACC; mariner transposon (Martin et al., 2000)

MP128      TACTAGCGACGCCCATCTATGTG; mariner transposon (Martin et al., 2000)

MP188      TTCATTTTCACCAACCAGGTAC; codY; +1032 This study

MP189      ATGGGCTGCTGAGTTTACTCCAG; codY; -618 This study
MP192  ggatccACGTCACTAAATAGCG;  *aliA*; -343  This study
MP193  CAGAAGCTTTCTGGTTTGTT;  *aliA*; -539  This study
MP194  TTGGAATTCCTCTTCTGGAAC;  *dexB*; +908  This study
MP195  ttagttgatgacgtggatccGCTTTTTATACAGTCCTCCC;  *dexB*; +1693  This study
rpsL_3  TGACATGGATACGGAAGTAG;  *rspL*; -798  This study
rpsL_4  ATGGTAAGCTGAGTTATAGC;  *rpsL*; +1204  This study

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\( \text{Ap}^R \), ampicillin resistance; \( \text{Cm}^R \), chloramphenicol resistance; \( \text{Kan}^R \), kanamycin resistance;
\( \text{Mtx}^R \), methotrexate resistance; \( \text{Spc}^R \), spectinomycin resistance; \( \text{Str}^R \), streptomycin resistance; \( \text{Trim}^R \), trimethoprim resistance.

\( \text{socY}^* \) refers to the presence of the \( \text{amiC}^{23} \), \( \text{amiC}^{A248C} \) or \( \text{amiC}^{G1459T} \) mutations as well as the \( \text{fatC}^{C496T} \) identified in this study and demonstrated to be required for tolerance of \( \text{codY} \) inactivation.

\( \text{socY} \) refers to the presence of the \( \text{amiC}^{G1459T} \) and \( \text{fatC}^{C496T} \) mutations.

Lowercase letters indicate nucleotide extensions to introduce convenient restriction sites (\( \text{NdeI}, \text{BamHI}, \text{EcoRI} \), respectively in \( \text{codYatg}, \text{codYstop} \), and MP192; underlined sequences) in the primers [Note that the \( \text{NdeI} \) site in \( \text{codYatg} \) introduced a \( \text{Ala} \to \text{Thr} \) change at the second aa position of \( \text{CodY} \)]. The start and stop codons of \( \text{codY} \) are shown in bold. In MP195, lowercase letters indicate nucleotide extension complementary to MP192.
Position is given with respect to the ATG of the corresponding gene; - and + indicate upstream and downstream, respectively.

C and A indicate respectively the co-transcribed and the reverse orientation of an inserted mini-transposon antibiotic resistance cassette with respect to the targeted gene.
**Fig. 1.** Insertion mutagenesis of *codY* is not possible in *S. pneumoniae* R6 unless the strain carries an ectopic complementing copy of the gene.

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

B. Placement of a second copy of *codY* under the control of the maltose-inducible P<sub>M</sub> promoter at CEP.

The inset in (B) shows control PCRs with the primer pair amiF1-AM40: lane 1, 2083 bp-long wildtype fragment; lane 2, 2760 bp-long CEP<sub>M</sub>-codY-kan fragment. M, kb ladder.

C. Recovery of mariner insertions at the *codY* locus in a recipient harboring a complementing ectopic copy.

The location of primers used to generate *codY* PCR fragments for mariner mutagenesis, MP188-MP189 (A) and codY1-codY2 (C) is indicated. Insertion sites were located through DNA sequencing at position -100 for kan<sup>90C</sup> (A) and -32, +163, +168, +300, and +365 for *spc<sup>5A</sup>, spc<sup>4C</sup>, spc<sup>3A</sup>, spc<sup>12A</sup>, and spc<sup>8A</sup>, respectively (C). Positions are given with respect to the first nucleotide of *codY* taken as +1; <sup>C</sup> and <sup>A</sup> indicate co-transcribed and reverse orientation of *spc* (*s*) or kan (*k*) cassette, respectively, with respect to *codY*. A CodY box (AATTTTCAGATAATT) previously identified in the promoter region of *codY* (Hendriksen *et al.*, 2008) is indicated as CYB. It is separated by 1 nt from the putative -35 promoter box of *codY*, itself separated by 17 nt from an extended -10 box with perfect match to the consensus (last nucleotide of this -10 located 26 nt upstream of the *codY* start).
Fig. 2. Integration of mariner minitransposons can occur at either site in the chromosome of codY<sup>+/+</sup> recipient cells.

A. PCR probing of the structure of an Spc<sup>R</sup> transformant (strain R2424) carrying the codY::spc<sup>3A</sup> minitransposon at the genuine codY locus using the following primer pairs:

codY1-codY2 (fragment/lane 2, 3787 bp), codY2-MP127 (fragment/lane 4, 1493 bp), MP127-codY1 (fragment/lane 3, 1196 bp), and amiF1-AM40 (fragment/lane 5, 1684 bp). Control PCR of the parental structure (strain R2350) using primer pairs codY1-codY2 (fragment/lane 1<sup>*</sup>, 2641 bp) and amiF1-kan1 (fragment/lane 6<sup>*</sup>, 1684 bp).

B. PCR probing of the structure of an Spc<sup>R</sup> transformant (strain R2425) carrying the codY::spc<sup>3A</sup> minitransposon at the CEP-codY locus using the following primer pairs: codY1-codY2 (fragment/lane E, 2641 bp), amiF1-kan1 (fragment/lane B, 2830 bp), amiF1-MP127 (fragment/lane C, 815 bp), and MP127-kan1 (fragment/lane D, 917 bp). Control PCR of the parental structure (strain R2350) using primer pairs codY1-codY2 (fragment/lane F<sup>*</sup>, 2641 bp) and amiF1-kan1 (fragment/lane A<sup>*</sup>, 1684 bp). M, kb ladder.

Fig. 3. Comparing transformation frequencies of codY<sup>spc3+/+</sup> in wildtype and codY<sup>+/+</sup> recipient cells.

Chromosomal DNA of strain R2438 (codY<sup>spc3+/+</sup> str4I) was used as donor. Strains used: isogenic codY<sup>+</sup> and codY<sup>+/+</sup> derivatives of R800, R6, and D39, respectively R895 and R2350, R6 and R2427, and D39 and TD73.
**Fig. 4.** Transformation data indicate the presence of two independent suppressor mutations or a chromosomal rearrangement in the previously described \textit{codY} mutant. Chromosomal DNA of strain TD81 (\textit{codY::spc}^{3A} \textit{socY str41}) was used as donor. Strains used: isogenic \textit{codY}^{+} and \textit{codY}^{+/-} derivatives of R800-\textit{hex}^{+}, R6, D39, and R800-\textit{hex}^{-}, respectively

R895 and R2350; R6 and R2427; D39 and TD73; R1818 and R2428.

**Fig. 5.** Effect of \textit{codY} inactivation and of \textit{socY} on spontaneous competence development. Pre-cultures prepared as described in the Experimental procedures were inoculated (1/50\textsuperscript{th} dilution) in C+Y medium (containing 300 µg ml\textsuperscript{-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: \textit{codY}^{+} TD82 (panels A-B); \textit{codY socY} mutant TD83 (panels C-D); \textit{socY} mutant TD96 (panels E-F).

**Fig. 6.** Inactivation of \textit{ami} and \textit{fat} results in tolerance of \textit{codY} inactivation.

A. Distribution of \textit{fatC} and \textit{amiC} mutations identified in strain D39\textit{ΔcodY} by whole genome sequence comparison. Positions are given with respect to the first nucleotide of \textit{fatC} (top) and \textit{amiC} (bottom), respectively, taken as +1. The location of the \textit{fatC::spc}^{23C} mariner insertion and \textit{amiC9} mutation used in panel B is also indicated.

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

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

Chromosomal DNA of strain TD80 (*codY::trim str41*) and TD81 (*codY::spc<sup>3A str41</sup>) was used as donor and Str<sup>R</sup> as well as, respectively, Trim<sup>R</sup> and Spc<sup>R</sup> transformants were scored. Strains used: wt, R246; *fatC::cat<sup>23C</sup>* mutant, R3002; *amiC9* mutant, R3003; *amiC9-fatC::cat<sup>23C</sup>* double mutant, R3004.

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

CodY and RitR share regulatory targets: CodY is a repressor of *ami, obl* and *fat-fec*, but activates *dpr*, which encodes an iron-storage peroxide resistance protein (Hendriksen *et al.*, 2008); RitR directly represses *fatD* (or *piuB*) and inactivation of *ritR* derepresses *amiC* while reducing *dpr* expression (Ulijasz *et al.*, 2004). We propose that inactivation of *codY* is not tolerated because it results in simultaneous derepression of iron uptake via Fat-Fec and possibly of heme utilization via Ami (see Discussion), as well as depletion of Dpr, which is required to protect cells from iron toxicity. ILV, isoleucine-leucine-valine (CodY activating branched chain aminoacids).
FIG. 1. Caymaris et al.
FIG. 2. Caymaris et al.

173x98mm (600 x 600 DPI)
FIG. 3. Caymaris et al.
FIG. 4. Caymaris et al.
FIG. 5. Caymaris et al.
FIG. 6. Caymaris et al.
FIG. 7. Caymaris et al.