Bacterial Olfaction

Reindert Nijland, J. Grant Burgess

► To cite this version:

Reindert Nijland, J. Grant Burgess. Bacterial Olfaction. *Biotechnology Journal*, 2010, 5 (9), pp.974. 10.1002/biot.201000174 . hal-00599454

HAL Id: hal-00599454

<https://hal.science/hal-00599454>

Submitted on 10 Jun 2011

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Bacterial Olfaction

Journal:	<i>Biotechnology Journal</i>
Manuscript ID:	biot.201000174.R1
Wiley - Manuscript type:	Short Communication
Date Submitted by the Author:	07-Jul-2010
Complete List of Authors:	Nijland, Reindert; Newcastle University, Dove Marine Laboratory; University Medical Center Utrecht, Medical Microbiology Burgess, J. Grant; Newcastle University, Dove Marine Laboratory
Primary Keywords:	Science Communication
Secondary Keywords:	Microbiology
Keywords:	Bacillus licheniformis, ammonia, olfaction

1 *Short communication*

2 **Bacterial Olfaction**

3
4 Reindert Nijland*, J. Grant Burgess[#]

5
6 Dove Marine Laboratory, School of Marine Science and Technology, Newcastle
7 University, Cullercoats, NE30 4PZ, United Kingdom

8
9 *) present address: Department of Medical Microbiology, University Medical
10 Center Utrecht, Utrecht, The Netherlands, r.nijland@umcutrecht.nl

11
12 Keywords: *Bacillus licheniformis*, ammonia, olfaction,

13
14 [#]) Corresponding author:

15 Professor J. Grant Burgess

16 Dove Marine Laboratory, School of Marine Science and Technology, Newcastle

17 University

18 North Shields

19 NE30 4PZ

20 United Kingdom

21 grant.burgess@ncl.ac.uk

22 Telephone: +44 191 222 6717

23 Fax: +44 191 222 5491

Abstract

Sensing their environment is a crucial ability of all life forms. In higher eukaryotes the sensing of airborne volatile compounds, or olfaction, is well developed. In plants, slime moulds and yeast there is also compelling evidence that these organisms can smell their environment and respond accordingly. Here we show that bacteria are also capable of olfaction. *Bacillus licheniformis* was able to sense airborne volatile metabolites produced by neighbouring bacterial cultures and cells could respond to this chemical information in a coordinated way. When *Bacillus licheniformis* was grown in a microtitre plate adjacent to a bacterial culture of the same or a different species, growing in complex medium, biofilm formation and pigment production were elicited by volatile molecules. A weaker response occurred in increasingly distant wells. The emitted volatile molecule was identified as ammonia. These data demonstrate that *B. licheniformis* has evolved the ability collect information about its environment from the surrounding air and physiologically respond to it in a manner similar to olfaction. This is the first time that a behavioural response triggered by odorant molecules received through the gas phase is described in bacteria.

1 In higher eukaryotes olfaction is an important sensory mechanism which provides
2 the organism with information about its environment. Olfaction is used to detect
3 the presence of food, predators and also other individuals of the same species.
4 Highly advanced olfactory systems are commonly found in animals, both
5 vertebrates and invertebrates (1). Plants can also detect volatile compounds and
6 respond accordingly (2, 3). Already in more primitive eukaryotes olfactory
7 systems are present, and it has been shown that *Dictyostelium* (4, 5) and yeast (6-
8 8) and can sense and respond to volatile chemical gradients such as ammonia. For
9 *Dictyostelium* it has been shown that the organism can produce ammonia
10 gradients and respond to such gradients by slug formation and migration (4, 5).
11 Separate yeast colonies communicate using ammonia pulses (6, 7), which results
12 in growth inhibition of the part of the colonies facing each other.

13 Cell-cell communication in bacteria is generally achieved using small
14 molecules, such as peptide pheromones, acylated homoserine lactones and other
15 low molecular weight natural products (9-12). Cell-cell communication has been
16 studied in bacteria which are in direct contact in biofilms, or most commonly, in
17 the liquid phase (9, 10). Bacteria are also known to produce a wide variety of
18 volatile metabolites and other chemicals including ammonia (13) through
19 overflow metabolism (14). Although bacteria are known to engage in chemical
20 communication with plants (15) and fungi (16) via gaseous molecules, to date
21 there have been no reports of intra-bacterial information exchange through the gas
22 phase. For bacteria the presence of a sensory system that detects volatile

1 compounds and which will inform bacteria of the presence of nearby competitors
2 or food sources would be highly advantageous.

3
4 We have been studying laboratory strains and marine isolates of *Bacillus*
5 *licheniformis* as a model system for understanding growth, communication and
6 competition within biofilms (17-19). We grew *Bacillus licheniformis* DSM13 for
7 2 days in complex medium (LB broth, Oxoid, UK) or biofilm specific medium
8 (MSgg-medium (20)) in clear 96 well flat bottom polystyrene tissue culture
9 microtitre plates (BD-Falcon, USA) with 200µl of culture per well with a closed
10 lid in a plastic container at 30°C. When growing *B. licheniformis* in this setup
11 using different growth media in different wells, we observed an unexpected
12 induction of biofilm formation and pigment production. This effect was
13 specifically observed in wells containing *B. licheniformis* in the biofilm
14 promoting medium MSgg (20), which were adjacent but physically distant to *B.*
15 *licheniformis* cultures growing in LB medium (Fig. 1). In wells directly next to
16 the inducer cultures, dense pellicle formation and pigment production was
17 elicited. A weaker response was observed in increasingly distant wells (Fig. 1,
18 Fig. 2).

19 To identify the signal that caused this pigmented pellicle (biofilm)
20 formation, biofilm induction experiments were performed with different growth
21 media and bacterial strains as the inducing cultures. *Bacillus subtilis*, *B.*
22 *licheniformis*, *Micrococcus luteus* and *Escherichia coli* were tested as inducers,
23 and all strains were able to induce a similar response in *B. licheniformis* grown in

MSgg medium. Generally, a response was observed when culture medium was complex and nutrient rich, or when a defined medium containing ammonium sulphate was used for growth of the emitting culture. No induction was observed if the sole nitrogen source in the inducing culture medium was glutamate. Two day old, stationary phase cultures and even sterile spent supernatant of nutrient rich cultures elicited the effect. Furthermore, after autoclaving the spent supernatant the induction was still observed. Lowering the pH of the inducing culture from pH 8 to pH 5 abolished the inducing effect (Fig 2A.), and increasing the pH to 9 increased the inducing effect (not shown). These observations indicated that the inducing compound was ammonia.

We confirmed that ammonia was present in all inducing cultures using Nessler's reagent (21). The presence of ammonia is not surprising, since bacterial metabolism is notoriously leaky (22) and when growing under high nutrient conditions many bacteria (13), including *B. licheniformis* (23), produce ammonia. The ammonia present in the aqueous solution is in equilibrium with ammonium ions (NH_4^+), depending on the pH of the solution. Since the NH_4^+ ion will not easily escape from solution, the airborne volatile NH_3 molecules are responsible for the observed effect. The ammonia dissolves on contact with the cells in suspension in the wells and an NH_3 - NH_4^+ equilibrium will be established.

The inducing effect of ammonia was subsequently confirmed by using a solution of ammonia to test for induction. We used the same microtitre plate setup, as

1 follows: *Bacillus licheniformis* DSM13 was grown in MSgg medium in the ten
2 central columns of a clear 96 well plate, stationary, for 24 hours, in a Fluostar
3 Optima plate reader (BMG labtech, UK). The cultures were flanked by one
4 column of wells containing an aqueous solution of ammonia at 25mM (left) and
5 2.5mM (right). Absorbance was measured at OD₅₉₅ every 30 minutes and the
6 average growth curve of 8 replicate wells was plotted. A 0.05% ammonia solution
7 in H₂O (25mM) showed a similar induction as observed previously with the
8 inducing cultures (Fig. 2B).

9 The nitrogen source in the recipient culture in MSgg medium was 34mM
10 glutamate. The use of an ammonium containing nitrogen source in the growth
11 medium would also lead to the release of volatile NH₃ into the gas phase. This
12 makes it difficult to distinguish between the inducing effect of the ammonium
13 present in the medium and volatile ammonia from itself or nearby cultures. To
14 circumvent this problem, and to obtain nitrogen surplus conditions we increased
15 the glutamate concentration of the recipient culture five fold to 170mM. In these
16 conditions, the biofilm inducing effect of volatile ammonia was still observed
17 (data not shown). This demonstrates that the recipient culture is able to sense and
18 respond to volatile ammonia even in the presence of excess nitrogen. We
19 conclude that volatile ammonia is an information carrying molecule in addition to
20 being able to provide nutritive value.

21 We have shown that *B. licheniformis* cells are capable of sensing other
22 bacteria growing nearby and of responding to this information. This may represent
23 the earliest evolutionary example of olfaction, since it is the biological detection

1 of a volatile compound over a distance. This observation suggests that the ability
2 to sense volatile signals and act on this information to enhance survival evolved
3 first in bacteria.

4 Ammonia is the simplest readily metabolizable nitrogen source. In many
5 environments, nitrogen is often one of the nutrients limiting for bacterial growth,
6 nitrogen use is strictly regulated (24, 25), and an ability to detect its nearby
7 presence would be advantageous. However, it was not observed until now that
8 bacteria can sense the presence of volatile ammonia and respond to this in a
9 coordinated manner.

10 Identification of the sensing mechanism and the signalling pathways
11 controlling subsequent biofilm formation is the next step in this line of research.
12 There is increasing evidence that Amt proteins identified in *E. coli* and a wide
13 range of bacteria including *Bacillus subtilis* act as ammonia sensors (26). It is also
14 interesting to note that proteomic data suggest that ammonia sensing may be
15 coupled to the regulation of a wide variety of environmental responses supporting
16 our observations that ammonia is an environmental signal molecule (26). The
17 ability of volatile molecules to affect bacterial growth and biofilm formation will
18 have major implications for our understanding of microbiology in general and of
19 biofilm biology specifically. A better understanding of the mechanisms that
20 trigger biofilm formation may also lead to new approaches to their prevention.
21 Indeed, the discovery of chemotaxis, quorum sensing and more recently
22 communication by physical contact (27) illustrates that bacteria appear to have
23 most of the five senses which include sight (responsiveness to light), touch

1 (contact dependent gene expression), taste (chemotaxis, Quorum Sensing) and
2 now smell. This new dimension to bacterial information exchange also opens up
3 the possibility of using this biochemical pathway as a model system for
4 understanding olfaction and the evolution of smell in higher organisms.

For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1 **Acknowledgments**

2 This research was funded by a by a fellowship from the European Community's
3 Seventh Framework Programme, under grant agreement PIEF-GA-2008-219592
4 to R.N., and a NERC follow-on fund Pathfinder grant to R.N. and J.G.B.
5 (NE/G011206/1). We would like to thank Mike Hall for advice on ammonia
6 chemistry.

7
8 The authors have declared no conflict of interest

For Peer Review

References

1. S. Firestein, How the olfactory system makes sense of scents. *Nature* 413, 211-218, 2001.
2. P. W. Pare, J. H. Tumlinson, Plant Volatiles as a Defense against Insect Herbivores. *Plant Physiol.* 121, 325-332, 1999.
3. H. S. Seo, J. T. Song, J.-J. Cheong, Y.-H. Lee, Y.-W. Lee, I. Hwang, J. S. Lee, Y. D. Choi, Jasmonic acid carboxyl methyltransferase: A key enzyme for jasmonate-regulated plant responses. *Proc Natl Acad Sci U S A* 98, 4788-4793, 2001.
4. C. K. Singleton, J. H. Kirsten, C. J. Dinsmore, Function of Ammonium Transporter A in the Initiation of Culmination of Development in *Dictyostelium discoideum*. *Eukaryotic Cell* 5, 991-996, 2006.
5. J. Schindler, M. Sussman, Ammonia determines the choice of morphogenetic pathways in *Dictyostelium discoideum*. *J Mol Biol* 116, 161-169, 1977.
6. Z. Palkova, B. Janderova, J. Gabriel, B. Zikanova, M. Pospisek, J. Forstova, Ammonia mediates communication between yeast colonies. *Nature* 390, 532-536, 1997.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

7. Z. Palkova, F. Devaux, M. Ricicova, L. Minarikova, S. Le Crom, C. Jacq, Ammonia Pulses and Metabolic Oscillations Guide Yeast Colony Development. *Mol. Biol. Cell* 13, 3901-3914, 2002.

8. M. Daniela, B. Simone, G. Maria Lodovica, G. Angelo, Volatile organic compounds: a potential direct long-distance mechanism for antagonistic action of *Fusarium oxysporum* strain MSA 35. *Environ Microbiol* 11, 844-854, 2009.

9. B. A. Lazazzera, A. D. Grossman, The ins and outs of peptide signaling. *Trends Microbiol.* 6, 288-294, 1998.

10. B. L. Bassler, R. Losick, Bacterially speaking. *Cell* 125, 237-246, 2006.

11. K. L. Visick, C. Fuqua, Decoding Microbial Chatter: Cell-Cell Communication in Bacteria. *J. Bacteriol.* 187, 5507-5519, 2005.

12. G. M. Dunny, The peptide pheromone-inducible conjugation system of *Enterococcus faecalis* plasmid pCF10: cell-cell signalling, gene transfer, complexity and evolution. *Philos Trans R Soc Lond B Biol Sci* 362, 1185-1193, 2007.

13. T. R. Whitehead, M. A. Cotta, Isolation and Identification of Hyper-Ammonia Producing Bacteria from Swine Manure Storage Pits. *Curr Microbiol* 48, 20-26, 2004.

- 1 14. J. B. Russell, G. M. Cook, Energetics of bacterial growth: balance of
2 anabolic and catabolic reactions. *Microbiol. Rev.* 59, 48-62, 1995.
- 3 15. C.-M. Ryu, M. A. Farag, C.-H. Hu, M. S. Reddy, H.-X. Wei, P. W. Paré, J.
4 W. Kloepper, Bacterial volatiles promote growth in *Arabidopsis*. *Proc Natl*
5 *Acad Sci U S A* 100, 4927-4932, 2003.
- 6 16. M. Kai, U. Effmert, G. Berg, B. Piechulla, Volatiles of bacterial
7 antagonists inhibit mycelial growth of the plant pathogen *Rhizoctonia*
8 *solani*. *Arch Microbiol* 187, 351-360, 2007.
- 9 17. L. Yan, K. G. Boyd, D. R. Adams, J. G. Burgess, Biofilm-specific cross-
10 species induction of antimicrobial compounds in *Bacilli*. *Appl Environ*
11 *Microbiol* 69, 3719, 2003.
- 12 18. P. Landini, D. Antoniani, J. G. Burgess, R. Nijland, Molecular
13 mechanisms of compounds affecting bacterial biofilm formation and
14 dispersal. *Appl Microbiol Biotechnol*, 2010.
- 15 19. R. Nijland, J. G. Burgess, J. Errington, J. W. Veening, Transformation of
16 environmental *Bacillus subtilis* isolates by transiently inducing genetic
17 competence. *PlosONE* 5 (3), 2010.
- 18 20. S. S. Branda, J. E. Gonzalez-Pastor, S. Ben-Yehuda, R. Losick, R. Kolter,
19 Fruiting body formation by *Bacillus subtilis*. *Proc Natl Acad Sci U S A* 98,
20 11621-11626, 2001.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1 21. A. I. Vogel, G. Svehla, Vogel's Textbook of Macro and Semimicro
2 Qualitative Inorganic Analysis (Longman, London, ed. 5th ed., 1979.
3 22. M. J. Church, The trophic tapestry of the sea. Proc Natl Acad Sci U S A
4 106, 15519-15520, 2009.
5 23. J. L. Meers, L. K. Pedersen, Nitrogen Assimilation by Bacillus
6 licheniformis Organisms Growing in Chemostat Cultures. J Gen Microbiol
7 70, 277-286 1972.
8 24. C. Detsch, J. Stulke, Ammonium utilization in Bacillus subtilis: transport
9 and regulatory functions of NrgA and NrgB. Microbiol 149, 3289-3297,
10 2003.
11 25. S. H. Fisher, L. V. Wray, Jr., Novel trans-Acting Bacillus subtilis glnA
12 Mutations That Derepress glnRA Expression. J Bacteriol 191, 2485-2492,
13 2009.
14 26. P. L. Tremblay, P. C. Hallenbeck, Of blood, brains and bacteria, the
15 Amt/Rh transporter family: emerging role of Amt as a unique microbial
16 sensor. Mol Microbiol 71, 12, (2009).
17 27. M. G. Blango, M. A. Mulvey, Bacterial landlines: contact-dependent
18 signaling in bacterial populations. Curr Opin Microbiol 12, 177-181, 2009.

Figure Legends

Figure 1: Induction of biofilm formation in microtitre plates

Bacillus licheniformis DSM13 was grown in LB (left six columns) or MSgg (right six columns) in clear 96 well flat bottom polystyrene tissue culture microtitre plate (BD-Falcon, USA) with 200µl of culture per well with a closed lid in a plastic container at 30°C for 2 days. The cultures in the MSgg medium responded to a volatile signal produced by the cultures growing in the LB medium, by forming a pigmented pellicle (biofilm) in a distance dependent manner. The lid was removed for the photograph. The image was taken using a Canon Powershot G6 digital camera, and in post processing the contrast of the total image was increased using Adobe Photoshop CS2.

Figure 2

2A: Effect of pH on the production of the volatile signal

Bacillus licheniformis DSM13 was grown stationary in MSgg medium at 30°C for 39 hours in the eight middle columns of a clear 96 well flat bottom polystyrene tissue culture plates in a Fluostar Optima plate reader (BMG labtech, UK). The cultures were flanked by two wells of cell free, spent supernatant of a *Bacillus licheniformis* DSM13 culture grown in LB (rows 1+2, which was at pH 8.0). On the right (rows 11+12) the same supernatant with the pH adjusted to 5.0 with HCl was used. Lowering the pH to 5.0 results in conversion of all free NH_3 to NH_4^+ , which is no longer volatile. As such no biofilm induction is observed. The image

1 was taken using a Canon Powershot G6 digital camera. Below: Absorbance of
2 these cells was measured at OD₅₉₅ every 30 minutes for 39 hours and the averaged
3 growth curve of 1 column/8 wells is shown. The absorbance at T = 39 hours is
4 visualized using a color scale ranging from OD₅₉₅ 0.85 (green) to OD₅₉₅ 1.70
5 (red).

6

7 **2B: confirmation of ammonia as the inducing signal**

8 *Bacillus licheniformis* DSM13 was grown stationary in MSgg medium in the ten
9 middle columns of a clear 96 well plate for 24 hours, in a Fluostar Optima plate
10 reader (BMG labtech, UK) at 30°C. The cultures were flanked by one column of
11 wells containing an aqueous solution of ammonia at 25mM (row 1) and 2.5mM
12 (row 12). Absorbance was measured at OD₅₉₅ every 30 minutes and the averaged
13 growthcurve of 1 column/8 wells was plotted. The absorbance at T = 24 hours
14 was visualized using a color scale ranging from OD₅₉₅ 0.70 (green) to OD₅₉₅ 1.35
15 (red).

ew

1 **Figure 1:**

Figure 2:

Figure 1: Induction of biofilm formation in microtitre plates

Bacillus licheniformis DSM13 was grown in LB (left six columns) or MSgg (right six columns) in clear 96 well flat bottom polystyrene tissue culture microtitre plate (BD-Falcon, USA) with 200 μ l of culture per well with a closed lid in a plastic container at 30°C for 2 days. The cultures in the MSgg medium responded to a volatile signal produced by the cultures growing in the LB medium, by forming a pigmented pellicle (biofilm) in a distance dependent manner. The lid was removed for the photograph. The image was taken using a Canon Powershot G6 digital camera, and in post processing the contrast of the total image was increased using Adobe Photoshop CS2.

Figure 2

2A: Effect of pH on the production of the volatile signal

Bacillus licheniformis DSM13 was grown stationary in MSgg medium at 30°C for 39 hours in the eight middle columns of a clear 96 well flat bottom polystyrene tissue culture plates in a Fluostar Optima plate reader (BMG labtech, UK). The cultures were flanked by two wells of cell free, spent supernatant of a *Bacillus licheniformis* DSM13 culture grown in LB (rows 1+2, which was at pH 8.0). On the right (rows 11+12) the same supernatant with the pH adjusted to 5.0 with HCl was used. Lowering the pH to 5.0 results in conversion of all free NH_3 to NH_4^+ , which is no longer volatile. As such no biofilm induction is observed. The image was taken using a Canon Powershot G6 digital camera. Below: Absorbance of these cells was measured at OD595 every 30 minutes for 39 hours and the averaged growth curve of 1 column/8 wells is shown. The absorbance at T = 39 hours is visualized using a color scale ranging from OD595 0.85 (green) to OD595 1.70 (red).

2B: confirmation of ammonia as the inducing signal

Bacillus licheniformis DSM13 was grown stationary in MSgg medium in the ten middle columns of a clear 96 well plate for 24 hours, in a Fluostar Optima plate reader (BMG labtech, UK) at 30°C. The cultures were flanked by one column of wells containing an aqueous solution of ammonia at 25mM (row 1) and 2.5mM (row 12). Absorbance was measured at OD595 every 30 minutes and the averaged growthcurve of 1 column/8 wells was plotted. The absorbance at T = 24 hours was visualized using a color scale ranging from OD595 0.70 (green) to OD595 1.35 (red).