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Cell Density-Regulated Recovery of Starved Biofilm Populations of Ammonia-Oxidizing Bacteria

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The speed of recovery of cell suspensions and biofilm populations of the ammonia oxidizer *Nitrosomonas europaea*, following starvation was determined. Stationary-phase cells, washed and resuspended in ammonium-free inorganic medium, were starved for periods of up to 42 days, after which the medium was supplemented with ammonium and subsequent growth was monitored by measuring nitrite concentration changes. Cultures exhibited a lag phase prior to exponential nitrite production, which increased from 8.72 h (no starvation) to 153 h after starvation for 42 days. Biofilm populations of *N. europaea* colonizing sand or soil particles in continuous-flow, fixed column reactors were starved by continuous supply of ammonium-free medium. Following resupply of ammonium, starved biofilms exhibited no lag phase prior to nitrite production, even after starvation for 43.2 days, although there was evidence of cell loss during starvation. Biofilm formation will therefore provide a significant ecological advantage for ammonia oxidizers in natural environments in which the substrate supply is intermittent. Cell density-dependent phenomena in a number of gram-negative bacteria are mediated by *N*-acyl homoserine lactones (AHL), including *N*-(3-oxohexanoyl)-L-homoserine lactone (OHHL). Addition of both ammonium and OHHL to cell suspensions starved for 28 days decreased the lag phase in a concentration-dependent manner from 53.4 h to a minimum of 10.8 h. AHL production by *N. europaea* was detected by using a *luxR-luxAB* AHL reporter system. The results suggest that rapid recovery of high-density biofilm populations may be due to production and accumulation of OHHL to levels not possible in relatively low-density cell suspensions.

Microorganisms in natural environments are subjected to temporal changes in environmental conditions and nutrient supply. Long-term survival therefore requires rapid response to environmental change. In particular, starved populations must be able to initiate metabolic activity when the substrate reappears to enable its use before removal by physical and chemical processes or utilization by competitors. This is particularly important for ammonia-oxidizing bacteria, as primary producers and heterotrophic bacteria, in both terrestrial and aquatic environments, compete for ammonium as a nitrogen source. The ammonia oxidizer *Nitrosomonas cryotolerans* has been shown to maintain viability during starvation (12, 13) but exhibits a lag period prior to recovery after supply of ammonium, which may be required for regeneration of reducing potential from ATP to initiate oxidation of ammonia to hydroxylamine (13).

Bacteria in natural environments commonly exist attached to particulate material, as biofilms, where their physiological properties may differ significantly from those of freely suspended cells. There is evidence, for example, that biofilm populations have greater resistance to antibiotics (9) and desiccation (5). Increased resistance to such factors may be due to production of extracellular polymeric material, which is frequently associated with biofilm populations (1), or to differences in the specific growth rate of organisms within biofilms (11). Differences in the physiology of biofilm and planktonic cells of nitrifying bacteria have also been observed by using experimental laboratory systems. For example, growth and activity of ammonia- and nitrite-oxidizing bacteria can be maintained in biofilms at pH values significantly lower than those required for growth of planktonic cells (2, 15). Nitrifier biofilms are also much more resistant to inhibitors of nitrification such as nitrapyrin (21) and potassium ethyl xanthate (26). These effects are frequently attributed to the production of extracellular polymeric substances (EPS) (16), which are a feature of ammonia oxidizer biofilms (10). The mechanistic basis for EPS protection is, however, not clear and, for ammonia-oxidizing bacteria, the chemical composition of EPS and its effects on physiology are difficult to characterize.

Although the physiology of lag phases prior to growth and of recovery from starvation is poorly understood, there is evidence that resuscitation of surface-associated populations occurs more rapidly than that of freely suspended cells. For example, Armstrong and Prosser (4) and Powell and Prosser (21) reported a reduction in the lag phase when the autotrophic ammonia oxidizer *Nitrosomonas europaea* was grown on the surface of clay minerals, in comparison with growth in liquid culture. This may be related to the phenomena of cell density signalling and bacterial communication, which are involved in resuscitation of *Micrococcus luteus* following starvation (27). Close proximity and high concentrations of cells may,

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therefore, provide an ecological advantage to organisms in natural environments subjected to an intermittent substrate supply. Such high concentrations are found in biofilms.

Density-dependent phenomena have recently been identified in a number of gram-negative bacteria (22, 25). These involve bacterial communication via a cell-cell signalling system and have provided new insights into the pathogenicity and virulence of human and plant pathogens. Termed quorum sensing, mechanisms as diverse as plasmid conjugation (28) and production of carbapenem (6, 7), exoenzymes (14, 19), and surfactants (18) have been shown to be dependent on the accumulation of signal molecules, *N*-acyl homoserine lactones (AHL). Here we identify an important advantage of biofilm formation by ammonia-oxidizing bacteria in providing rapid recovery following starvation, and we provide evidence that recovery is associated with production, at high cell density, of an AHL. This implicates AHL in the control of an aspect of primary metabolism fundamental to the growth and activity of bacteria in natural environments.

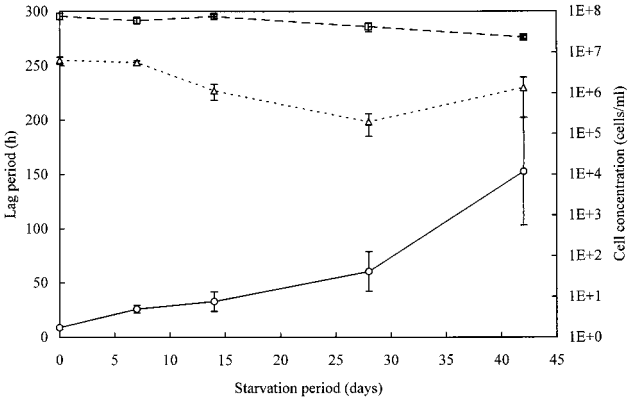

FIG. 1. Changes in total cell concentration (□), viable cell concentration (△), and lag period length prior to exponential nitrite production (○) following starvation of *N. europaea* in ammonium-free mineral salts medium. Values are means, and error bars represent SEs.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *N. europaea*, originally isolated from soil, was supplied by R. M. Macdonald, Rothamsted Experimental Station, Harpenden, Hertfordshire, United Kingdom. Growth and maintenance were carried out as described by Keen and Prosser (15) in Skinner and Walker (SW) (23) medium containing 50 μg of NH_4^+-N ml^{-1} as ammonium sulfate and modified as described by Powell and Prosser (20).

Starvation and recovery of cell suspensions. *N. europaea* was grown in 1-liter flasks containing 800 ml of SW medium with 100 μg of NH_4^+-N ml^{-1}. Cells were harvested during the late exponential phase and resuspended in ammonium-free SW medium (AFSW) to give a final concentration of ca. 10^9 cells ml^{-1}. This suspension was then used to inoculate 100 ml of AFSW in 250-ml Erlenmeyer flasks to give a final cell concentration of ca. 10^7 cells ml^{-1}. Starved cell suspensions were incubated at 25°C in the dark for up to 42 days and were sampled for total cell enumeration by using a Helber counting chamber. Flasks were weighed regularly, and water lost by evaporation was replaced by aseptic addition of sterile distilled water. To assess recovery, 10-ml samples of starved cell suspensions were dispensed into sterile Universal bottles and 100 μl of an ammonium sulfate solution was added to give a final concentration of 50 μg of NH_4^+-N ml^{-1}. Cultures were incubated with shaking at 30°C, and growth was assessed by ammonium and nitrite concentration analysis of 1-ml samples until ammonium oxidation was limited by pH reduction. Ammonium and nitrite were analyzed by using an Alpkem RFA-300 Rapid Flow Analyser. The lag period was determined by extrapolation of linear regions of semilogarithmic plots of nitrite concentration versus time during exponential-phase nitrite production. Each analysis of recovery was carried out in duplicate, and the entire experiment was duplicated. Total cell concentrations of starved cell suspensions were determined by using a Helber counting chamber. Viable cell concentrations were determined by using the most-probable-number method, with decimal dilutions of samples and inoculation of 50 μl from each dilution of five test tubes containing 450 μl of SW medium with 50 μg of NH_4^+-N ml^{-1}. Tubes were incubated at 30°C in the dark for 6 weeks, and growth was assessed as production of acid and nitrite and disappearance of ammonium.

Starvation and recovery of biofilm populations. Biofilms were prepared by growth of *N. europaea* in continuous-flow soil and sand columns. Each column consisted of a plastic cylinder with an internal diameter of 4 cm and a length of 40 cm that was closed at each end with a silicone rubber stopper. Soil columns were packed with 40 g of a mixture of Craibstone soil (Countesswells series; sandy loam; cation exchange capacity, 7.4 cmol kg^{-1}; organic matter, 4.25%), acid-washed fine mesh sand, and $Ca(OH)_2$ at a ratio of 60:3.2:0.31 to give a final pH value of 8.4. Sand columns were packed with 40 g of acid-washed fine mesh sand. Filter-sterilized air and medium were supplied to the column through two Pasteur pipettes inserted through the stopper at the top of the column. Effluent was collected through a Pasteur pipette inserted through the stopper at the base of the column. Even distribution of liquid reaching the sand or soil surface was achieved by placing a microfiber filter (Whatman) on the upper surface of the soil or sand. Sand and soil columns were autoclaved once and three times, respectively, at 121°C for 30 min with connecting tubing in place.

After autoclaving and cooling, SW medium (with phenol red omitted) was passed through the column for 5 days prior to inoculation with 15 ml of a late-exponential-phase culture of *N. europaea*. The column was incubated in a batch for 7 days at 30°C, after which SW medium (50 μg of NH_4^+-N ml^{-1}, pH 8) was supplied at a constant flow rate of 2 ml h^{-1} by using an LKB 2132 Microperpex peristaltic pump. Effluent from the base of the column was collected in an LKB 2070 Ultrorac II fraction collector. Potassium ethyl xanthate, a nitrification inhibitor, was added to samples to a final concentration of 20 μg ml^{-1} to prevent activity in the samples prior to analysis for ammonium and nitrite as described above.

Following development of an active and stable biofilm, as indicated by the steady states of the ammonium and nitrite concentrations in the effluent from the column, the population was starved by continuous supply to the column of AFSW in place of SW. Establishment of steady states was determined as described by Keen and Prosser (15). After the required period of starvation, SW was supplied and recovery was monitored by measuring increases in the nitrite concentration in the effluent from the column.

Detection of AHL production by *N. europaea*. *N. europaea* was grown in a batch culture in SW medium containing 50 μg of NH_4^+-N ml^{-1} as described above. Spent medium was extracted twice with 2 volumes of chloroform and evaporated to a dry powder with a rotary evaporator. The residue was redissolved in 100 μl of ethyl acetate and separated further by using a DCC Silica column with an eluent of pentane. Fractions (10 ml) were collected, evaporated to dryness, and redissolved in 100 μl of ethyl acetate. A 5-μl volume of each fraction was added to 100 μl of Luria-Bertani (LB) broth in wells of microtiter plates. Doubling dilutions were then prepared by addition of 100-μl samples in 100 μl of LB broth. A 100-μl volume of an overnight culture of *Escherichia coli* carrying a recombinant AHL reporter system (24) was added to each well, and plates were incubated overnight at 30°C. Luminescence was detected and quantified by using a Hamamatsu Argus 100 Vim 3 Photon Video Camera. *N*-(3-Oxohexanoyl)-L-homoserine lactone (OHHL) was synthesized as described by Chhabra et al. (8). Controls were LB broth and ethyl acetate.

Effect of OHHL on the lag phase following recovery of starved cells. Early stationary-phase cells were harvested, washed three times, and resuspended in AFSW (100 ml) in 250-ml Erlenmeyer flasks at a final concentration of ca. 10^7 cells ml^{-1}. Cell suspensions were incubated for 28 days at 30°C in the dark. The effect of OHHL on the length of the lag period was determined by addition of ammonium sulfate and OHHL (final concentrations, 50 μg of NH_4^+-N ml^{-1} and 0 to 2 μg ml^{-1}, respectively). Cultures were incubated with shaking at 30°C, and growth was monitored by determining changes in nitrite concentration. The lag period was determined as described above.

RESULTS

Recovery of starved cell suspensions. Washed cells of *N. europaea* were suspended in AFSW and starved for up to 42 days. Addition of ammonium was followed, after a lag phase, by an exponential increase in nitrite concentration, reflecting an exponential increase in biomass concentration. The lag phase prior to the exponential increase in the nitrite concentration increased as the starvation period increased (Fig. 1). Cells had a mean lag phase of 8.72 h (standard error [SE], 1.54 h) immediately after resuspension in starvation medium. This increased to a mean of 153 h (SE, 50 h) after starvation for 42 days. The total cell concentration decreased from 7.56×10^7 to 2.43×10^7 cells ml^{-1} during the 42-day incubation period. Total cell concentrations at days 0 and 42 were significantly different ($P = 0.03$) as determined by Student's t test, and a one-way analysis of variance of data at each sampling

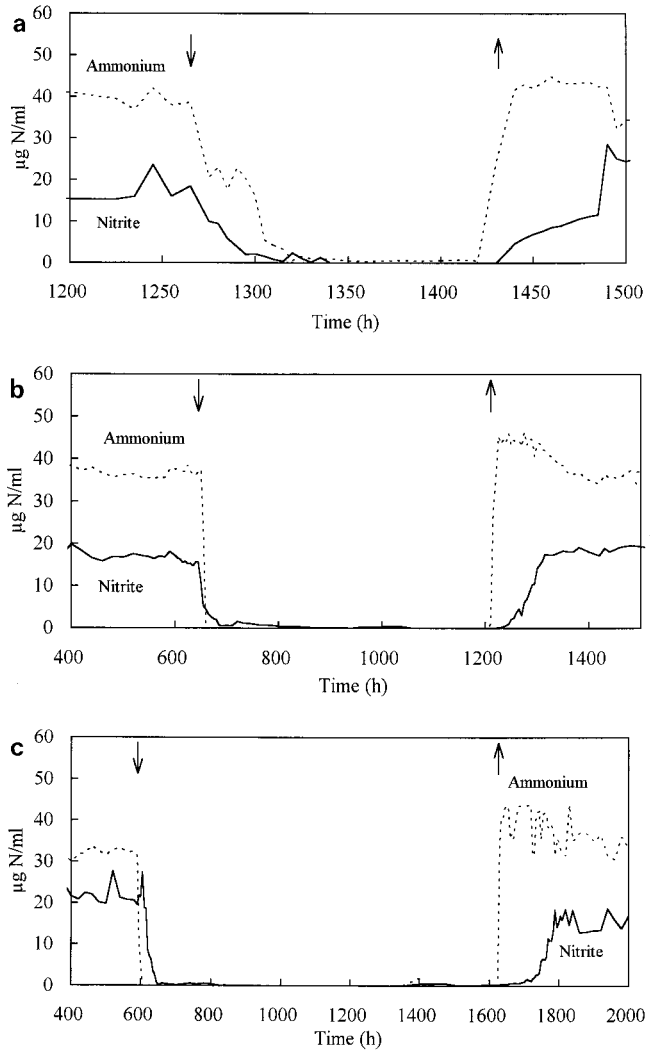

FIG. 2. Changes in ammonium (– – –) and nitrite (——) concentrations in effluent from continuous-flow sand columns supplied with mineral salts medium containing ammonium or ammonium-free medium. The arrows indicate the times at which ammonium was supplied (upward arrows) and removed (downward arrows). Data are from three columns operated under identical conditions but with biofilms starved for 7.7 (a), 23 (b), and 42 (c) days. Samples were taken at 5-h intervals.

point also indicated a significant decrease in the total cell concentration ($P = 0.005$). Viable cell concentrations at days 0 and 42 were not significantly different ($P = 0.14$, Student t test), and analysis of variance indicated no effect of time at the 5% level of significance ($P = 0.066$). The difference in significance associated with total and viable cell concentrations reflects the greater variability associated with most-probable-number viable cell counts, as demonstrated by the greater SE values (Fig. 1).

Recovery of biofilm populations. Recovery of starved cell suspensions was compared with that of biofilm populations formed in three continuous-flow sand columns and one soil column. Ammonium or ammonium-free medium was supplied continuously at a rate of 2 ml h^{-1}, providing a dilution rate significantly greater than the maximum specific growth rate (μ_{max}) of *N. europaea*. Activity, measured as changes in ammonium and nitrite concentrations in the effluent from the column, was therefore due almost entirely to that of biofilm cells rather than freely suspended cells, which had been rapidly washed out. Steady-state ammonium and nitrite concentrations were established in sand column effluents, after which ammonium-free medium was supplied for periods of 7.7, 22.7, 23.5, and 43.2 days prior to resupply with complete medium. Changes in effluent ammonium and nitrite concentrations are represented in Fig. 2 for starvation for periods of 7.7, 22.7, and 43.2 days.

Following supply of AFSW, ammonium and nitrite concentrations decreased to background levels within ca. 50 h as the ammonium in the interstitial water was converted to nitrite and washed out of the column. Ammonium and nitrite concentrations then remained at background levels until supply of complete medium, SW. Following supply of ammonium to cells starved for 7.7 days, ammonium and nitrite were detected simultaneously in the column, indicating immediate recovery of ammonium oxidation (Fig. 2a). The increase in the nitrite concentration was not exponential, and a new steady state was established within 60 h (Table 1). These kinetics indicate that the population size did not change significantly during starvation, although all of the biomass did not become active immediately. Resupply of ammonium to a biofilm starved for 22.7 days (Fig. 2b) led to detection of nitrite less than 25 h after the appearance of ammonium in the column effluent. Following a second starvation period of 23 days (data not shown), nitrite was detected prior to ammonium. In both cases, the nitrite concentration increased exponentially, at specific rates of 0.085 and 0.037 h^{-1}, and steady states were established within 95 and 110 h at concentrations similar to those prior to starvation (Fig. 2b and Table 1). Cells starved for 43 days also recovered immediately following resupply of ammonium with the simultaneous appearance of ammonium and nitrite in the column effluent and an exponential increase in the nitrite concentration. The nitrite concentration increased at a specific rate of 0.020 h^{-1}, and a steady state was re-established after 170 h.

A similar experiment was carried out by using a continuous-flow soil column. Following establishment of steady-state ammonium and nitrite concentrations, in the effluent, ammonium-free medium was supplied. Ammonium concentrations decreased, but at a much lower rate than in the sand columns described above (Fig. 3). This is believed to be due to the greater cation-exchange capacity of soil and to the presence of smaller pores, increasing the time for removal and conversion of ammonium in interstitial water. Ammonium and nitrite concentrations reached background levels 18 days after supply of ammonium-free medium. After a further 13 days of starvation, ammonium was supplied, leading to immediate increases in effluent ammonium and nitrite concentrations. The nitrite concentration increase was initially greater than that of ammonium and occurred exponentially at a specific rate of 0.015 h^{-1}. New steady states of ammonium and nitrite, similar to those before starvation, were established after 140 h.

TABLE 1. Recovery of starved biofilm populations in sand and soil columns following resupply of ammonium

Starvation period (days)	Specific increase in nitrite concn (h^{-1})	Time before establishment of new steady state (h)
7.7	Nonexponential increase	60
22.7	0.085	95
23.5	0.037	110
43.2	0.020	170
32 (13)[a]	0.015	140

[a] The value in parentheses is the starvation period following the disappearance of ammonium in effluent from the column.

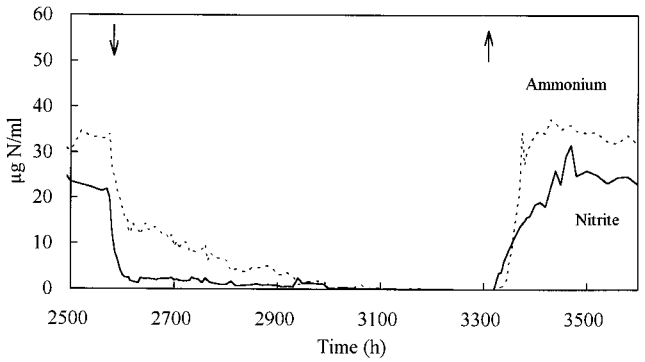

FIG. 3. Changes in ammonium (– – –) and nitrite (——) concentrations in effluent from a continuous-flow soil column supplied with mineral salts medium containing ammonium or ammonium-free medium. The arrows indicate the times at which ammonium was supplied (upward arrows) and removed (downward arrows). Samples were taken at 5-h intervals.

Effect of OHHL on recovery. The effect of synthetic OHHL on the lag phase prior to regrowth of starved cells was determined by simultaneous addition of both ammonium and OHHL to low-density (10^7 cell ml^{-1}) suspensions of *N. europaea* starved for 28 days in ammonium-free medium (Fig. 4). In the absence of OHHL, the lag period was 53.4 h (SE, 1.14), which is similar to that described above for a similar starvation period (Fig. 1). Addition of OHHL reduced the length of the lag phase in a classic dose-response curve. The lowest concentration of OHHL investigated (0.01 μg ml^{-1}) decreased the lag phase dramatically to 22.2 h (SE, 3.42), with a further reduction to 10.8 h (SE, 0.96) at 0.1 μg ml^{-1}. Higher concentrations resulted in smaller reductions in the lag phase, but all concentrations reduced the lag phase markedly in comparison with the control.

Production of AHL by *N. europaea*. The ability of *N. europaea* to produce homoserine lactones was investigated by using a recombinant *E. coli* reporter system. Spent medium from *N. europaea* cultures was extracted with chloroform and fractionated on a silica column (7). Dilutions of each fraction were mixed with the reporter strain and LB broth, and after incubation, luminescence was detected and quantified by using a Photon Video Camera (Fig. 5). Controls containing LB broth or ethyl acetate gave luminescence values of 0.673 and 0.655 relative light units (RLU), respectively, and test samples gave values ranging from 306 RLU (no dilution) to 8.8 RLU at the 1/512 dilution.

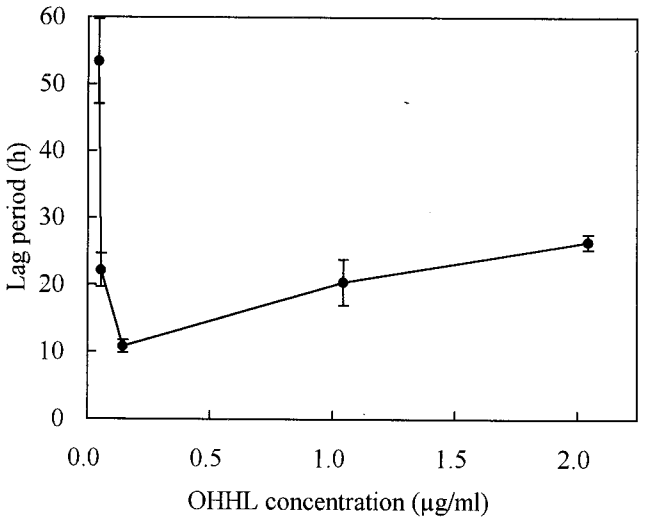

FIG. 4. Effect of OHHL on the lag phase prior to the growth of *N. europaea* cells starved in ammonium-free medium for 28 days. The values are means for triplicate flasks, and the error bars indicate SEs.

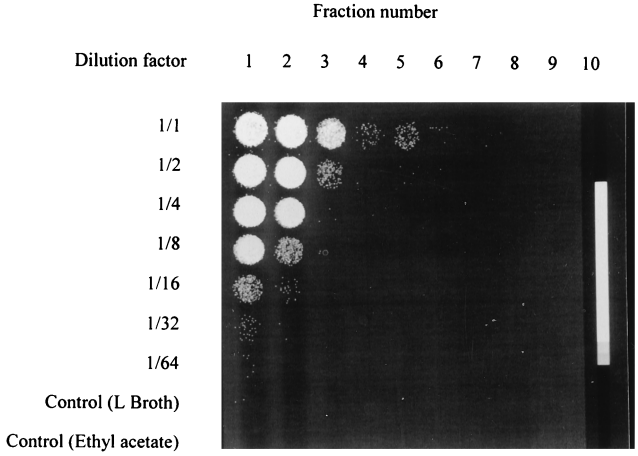

FIG. 5. Detection of AHL production by *N. europaea*. Doubling dilutions of a chloroform extract of spent medium from an overnight culture of *N. europaea* were mixed with 100 μl of an overnight culture of a *luxR-luxAB* AHL reporter system. Controls were LB broth and ethyl acetate.

DISCUSSION

Growth rates and lag phases of ammonia oxidizers were measured in this study on the basis of exponential increases in the product (nitrite) concentration, rather than the biomass concentration. A disadvantage of this approach is that apparent lag phases depend on the delay prior to initiation of metabolic activity and growth and/or on the cell concentration required for detection of activity. During starvation experiments, therefore, an increase in the measured lag phase can be due to a real effect on the onset of growth and/or a reduction in the cell concentration. The approximately threefold reduction in the total cell concentration during starvation in cell suspensions for 42 days would potentially increase the lag period by the time required to increase the cell concentration by this amount after recovery, i.e., $\log_e 3/\mu_{max}$. For *N. europaea* growing under the conditions employed in this study, μ_{max} was 0.06 h^{-1}. The reduction in the cell concentration at 42 days would therefore lead to an increase in the apparent lag of no more than ca. 18 h, which is significantly less than the observed lag period of 150 h. The effect on the lag phase was therefore a real effect of starvation and similar to that observed by other workers (3, 17).

A lag period of 150 h (6 days) for ammonia oxidizers in natural environments would be of considerable significance. Nitrogen is often a limiting nutrient in aquatic and terrestrial environments, and competition for ammonium from heterotrophs and phototrophs (plants and microorganisms) will consequently be intense. Ammonium is therefore unlikely to remain available at high concentrations for several days in such environments, and a lag phase of 150 h will seriously reduce the probability of its use for growth by ammonia oxidizers.

In contrast to cell suspensions, there was no evidence of a lag phase in either the activity or the growth of starved biofilm populations of ammonia oxidizers. In sand columns, nitrite and ammonium appeared simultaneously in column effluent, even

after starvation for 43.2 days. In the soil column, nitrite appeared at a greater initial rate than ammonium as a result of retardation of ammonium due to adsorption by soil particles. Two aspects of recovery indicate that cells were lost from biofilms during starvation through a combination of cell death, detachment, and washout. The first is the time taken to establish new steady states of ammonium and nitrite. This time increased with the starvation period, from 60 h after starvation for 7.7 days to 170 h after starvation for 43.2 days, suggesting that the decrease in population size occurred continuously during the starvation period. The second is the kinetics of nitrite production. With the exception of the shortest starvation period, the nitrite concentration increased exponentially immediately following resupply of ammonium. The specific nitrite concentration increases, equivalent to the μ_{max}, were within the range expected for this strain of *N. europaea* but were affected by factors within the biofilm, e.g., oxygen supply and a balance between cell growth and cell loss through washout. An exponential increase in the nitrite concentration was not observed after starvation for only 7.7 days, suggesting that population size had not decreased significantly during the starvation period and that the maximum population re-established full activity rapidly after supply of ammonium.

The data therefore indicate rapid recovery of both the activity and the growth of starved biofilm populations. While recovery of activity without a lag phase has been observed for other organisms in cell suspensions, the absence of a lag period prior to growth of *N. europaea* appears to result from surface attachment and biofilm formation. This confirms the observations of shortened lag periods for ammonia-oxidizing bacteria adsorbed to clay minerals (4, 21). A number of biofilm properties can be invoked to explain this reduction in the lag phase. For example, anionic components of EPS may adsorb ammonium, which may then be released gradually within the biofilm, maintaining cells at low levels of activity. The potential duration of this type of effect was illustrated during starvation of the biofilm in the soil column, when ammonium was released from soil particles long after the ammonium supply had been halted.

The effects of OHHL suggest an alternative explanation which we were able to test experimentally, i.e., that *N. europaea* produces an AHL. Addition of OHHL to suspended cells at a low cell density produced a significant decrease in the lag phase, from 53.4 to 10.8 h, after starvation for 28 days. The concentration of OHHL which gave the greatest reduction in the lag phase is 10-fold greater than the optimum for bioluminescence induction in *Photobacterium fischeri* but 10-fold less than that required for optimum carbapenem production in *Erwinia carotovora* (7). *N. europaea* therefore responded to concentrations of OHHL similar to those affecting previously characterized cell density-dependent secondary metabolic processes.

A reduction in the lag phase of more than 40 h prior to growth is a startling response to the addition of a small chemical signalling molecule and is the first demonstration of an effect of AHL on a primary metabolic process. It is clear that under the conditions of normal growth in liquid culture, cell densities never reach those which enable accumulation of AHL to physiologically effective levels. This raises the following question: under what growth conditions does quorum sensing operate in *N. europaea*? Although detectable in culture supernatants after the extraction and concentration procedures described above, AHL could not be detected directly by the *lux E. coli* AHL reporter, even in late-exponential-phase liquid cultures. If, however, *N. europaea* cells were concentrated, by centrifugation, to 10^{10} cells ml^{-1} and incubated in the presence of ammonium, high levels of AHL could be detected by using the reporter strain.

Cell densities of 10^{10} ml^{-1} occur only in biofilms and cell aggregates, and if AHL is produced, its diffusion from cells is reduced in biofilms. It is possible, therefore, that the previously observed reduction in the lag period was due to AHL production and accumulation immediately after activation of starved cells with ammonium. These results are evidence that cell density signalling, possibly mediated by AHL, has a direct role in activating cell growth in biofilms and may be responsible for other properties of biofilms that distinguish them from suspended cells at low density. The ecological significance of the results described here lies in the ability of high cell densities to respond immediately to increased concentrations of nutrients. In the presence of competitors, organisms that exhibit a long lag phase will be disadvantaged. This applies particularly to ammonia oxidizers, which suffer competition for ammonium from primary producers in aquatic environments and from plants and heterotrophic microorganisms in terrestrial environments.

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REFERENCES

1. **Allison, D. G., and M. J. Matthews.** 1992. Effect of polysaccharide interactions on antibiotic susceptibility of *Pseudomonas aeruginosa*. Biofouling **4:**243.
2. **Allison, S. M., and J. I. Prosser.** 1993. Ammonia oxidation at low pH by attached populations of nitrifying bacteria. Soil Biol. Biochem. **25:**935–941.
3. **Amy, P. S., C. Pauling, and R. Y. Morita.** 1983. Recovery from nutrient starvation by a marine *Vibrio* sp. Appl. Environ. Microbiol. **45:**1685–1690.
4. **Armstrong, E. F., and J. I. Prosser.** 1988. Growth of *Nitrosomonas europaea* on ammonia-treated vermiculite. Soil Biol. Biochem. **20:**409–411.
5. **Atkins, E. D. T., D. H. Isaac, and H. F. Elloway.** 1979. Conformations of microbial extracellular polysaccharides by X-ray diffraction: progress on the *Klebsiella* serotypes, p. 161–189. *In* R. C. W. Berkeley, G. W. Gooday, and D. C. Ellwood (ed.), Polysaccharides and polysaccharases. Academic Press, London, England.
6. **Bainton, N. J., B. W. Bycroft, S. R. Chhabra, P. Stead, L. Gledhill, P. J. Hill, C. E. D. Rees, M. K. Winson, G. P. C. Salmond, G. S. A. B. Stewart, and P. Williams.** 1992. A general role for the *lux* autoinducer in bacterial cell signalling: control of antibiotic biosynthesis in *Erwinia*. Gene **116:**87–91.
7. **Bainton, N. J., P. Stead, S. R. Chhabra, B. W. Bycroft, G. P. C. Salmond, G. S. A. B. Stewart, and P. Williams.** 1992. *N*-(3-Oxohexanoyl)-L-homoserine lactone regulates carbapenem antibiotic production in *Erwinia carotovora*. Biochem. J. **288:**997–1004.
8. **Chhabra, S. R., P. Stead, N. J. Bainton, G. P. C. Salmond, G. S. A. B. Stewart, P. Williams, and B. W. Bycroft.** 1993. Autoregulation of carbapenem biosynthesis in *Erwinia carotovora* by analogues of n-3 oxohexanoyl-l-homoserine lactone. J. Antibiot. **46:**441–454.
9. **Costerton, J. W., R. T. Irvin, and K. J. Cheng.** 1981. The bacterial glycocalyx in nature and disease. Annu. Rev. Microbiol. **35:**299–324.
10. **Cox, D. J., M. J. Bazin, and K. Gull.** 1980. Distribution of bacteria in a continuous-flow nitrification column. Soil Biol. Biochem. **12:**241–246.
11. **Gilbert, P., P. J. Collier, and M. R. W. Brown.** 1990. Influence of growth rate on susceptibility to antimicrobial agents: biofilms, cell cycle, dormancy, and stringent response. Antimicrob. Agents Chemother. **34:**1865–1868.
12. **Johnstone, B. H., and R. D. Jones.** 1988. Recovery of a marine chemolithotrophic ammonium-oxidising bacterium from long term energy source deprivation. Can. J. Microbiol. **34:**1347–1350.
13. **Jones, R. D., and R. Y. Morita.** 1985. Low temperature growth and whole cell kinetics of a marine ammonium oxidiser. Mar. Ecol. Prog. Ser. **21:**239–243.
14. **Jones, S., B. Yu, N. J. Bainton, M. Birdsall, B. W. Bycroft, S. R. Chhabra, A. J. R. Cox, P. Golby, P. J. Reeves, S. Stephens, M. K. Winson, G. P. C. Salmond, G. S. A. B. Stewart, and P. Williams.** 1993. The *lux* autoinducer regulates the production of exoenzyme virulence determinants in *Erwinia carotovora* and *Pseudomonas aeruginosa*. EMBO J. **12:**2477–2482.
15. **Keen, G. A., and J. I. Prosser.** 1987. Interrelationship between pH and surface growth of *Nitrobacter*. Soil Biol. Biochem. **19:**665–672.
16. **Marshall, K. C., R. Stout, and R. Mitchell.** 1971. Mechanism of the initial events in the sorption of marine bacteria to surfaces. J. Gen. Microbiol. **68:**337–348.
17. **Meikle, A., L. A. Glover, K. Killham, and J. I. Prosser.** 1994. Potential

luminescence as an indicator of activation of genetically modified *Pseudomonas fluorescens* in liquid culture and in soil. Soil Biol. Biochem. **26:**747–755.

18. **Ochsner, U. A., A. K. Koch, A. Fiechter, and J. Reiser.** 1994. Isolation and characterization of a regulatory gene affecting rhamnolipid biosurfactant synthesis in *Pseudomonas aeruginosa*. J. Bacteriol. **176:**2044–2054.
19. **Pirhonen, M., D. Flego, R. Heikinheimo, and E. T. Palva.** 1993. A small diffusible signal molecule is responsible for the global control of virulence and exoenzyme production in the plant pathogen *Erwinia carotovora*. EMBO J. **12:**2467–2476.
20. **Powell, S. J., and J. I. Prosser.** 1985. The effect of nitrapyrin and chloropicolinic acid on ammonium oxidation by *Nitrosomonas europaea*. FEMS Microbiol. Lett. **28:**51–54.
21. **Powell, S. J., and J. I. Prosser.** 1991. Protection of *Nitrosomonas europaea* colonising clay minerals from inhibition by nitrapyrin. J. Gen. Microbiol. **137:**1923–1929.
22. **Salmond, G. P. C., B. W. Bycroft, G. S. A. B. Stewart, and P. Williams.** 1995. The bacterial 'enigma': cracking the code of cell-cell communication. Mol. Microbiol. **16:**615–624.
23. **Skinner, F. A., and N. Walker.** 1961. Growth of *Nitrosomonas europaea* in batch and continuous culture. Arch. Mikrobiol. **38:**339–349.
24. **Swift, S., M. K. Winson, P. F. Chan, N. J. Bainton, M. Birdsall, P. J. Reeves, C. E. D. Rees, S. R. Chhabra, P. J. Hill, J. P. Throup, B. W. Bycroft, G. P. C. Salmond, P. Williams, and G. S. A. B. Stewart.** 1993. A novel strategy for the isolation of *luxI* homologues: evidence for the widespread distribution of a luxR:luxI superfamily in enteric bacteria. Mol. Microbiol. **10:**511–520.
25. **Swift, S., J. P. Throup, G. P. C. Salmond, and G. S. A. B. Stewart.** 1996. Quorum sensing: a population density component in the determination of bacterial phenotype. Trends Biochem. Sci. **21:**214–219.
26. **Underhill, S. E., and J. I. Prosser.** 1987. Surface attachment of nitrifying bacteria and their inhibition by potassium ethyl xanthate. Microb. Ecol. **14:**129–139.
27. **Votyakova, T. V., A. S. Kaprelyants, and D. B. Kell.** 1994. Influence of viable cells on the resuscitation of dormant cells in *Micrococcus luteus* cultures held in an extended stationary phase: the population effect. Appl. Environ. Microbiol. **60:**3284–3291.
28. **Zhang, L., P. J. Murphy, A. Kerr, and M. E. Tate.** 1993. *Agrobacterium* conjugation and gene regulation by *N*-acyl-L-homoserine lactone. Nature **362:**446–448.