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Increase in Spermine Content Coordinated with Siderophore Production in *Paracoccus denitrificans*

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Spermine is present in relatively low amounts in *Paracoccus denitrificans* cultured aerobically in an ammonium succinate minimal salts medium supplemented with 50 μM iron(III). However, in iron-deprived cultures [minimal salts medium containing 0.5 μM iron(III)], spermine content increases by an order of magnitude in coordination with the well-known responses to iron deprivation, e.g., derepression of siderophore synthesis and siderophore excretion. When iron-deprived cultures exhibiting both high spermine content and strong siderophore production are reseeded into fresh minimal salts medium containing 50 μM iron[III], both siderophore production and spermine content fall rapidly. Five hours after iron supplementation, spermine is below limits of detection. These results suggest a specific role for spermine in the response of *P. denitrificans* to low-iron stress.

Several years ago (20, 37, 40, 41, 43), the conventional wisdom was that, of the three prototypical polyamines, only the diamine putrescine (PUT) and the triamine spermidine (SPD) occurred in prokaryotes. The tetraamine, spermine (SPM), was in general considered an innovation of eukaryotes. A wide variety of unusual polyamines, most homologs of the three prototypes (e.g., norspermidine and homospermidine), have been isolated from various microrganisms (12, 17, 21, 22, 24, 25, 31), and recently polyamine analysis has proven to be a valuable tool for chemotaxonomic classification of *Eubacteria* and *Archaebacteria* species (10, 19, 38). These data do reveal the presence of SPM, most notably, in a number of thermophilic species of eubacteria as well as archaebacteria (2, 9, 18, 27, 34, 35). The amounts found in most nonthermophiles often seem too small to suggest an important function for SPM in these species. However, many of these experiments involve cultures grown in complex, sometimes ill-defined media, making interpretation of the literature more difficult. Thus, there are several different reports of the presence of small amounts of SPM in *Paracoccus denitrificans* (2, 23). While iron-replete cultures of this microorganism have very low SPM content, we now report a striking increase in SPM content in response to iron deprivation.

MATERIALS AND METHODS

All experiments were conducted aerobically in a chemically defined ammonium-succinate minimal salts liquid medium. To obtain reproducible growth curves and siderophore production for *P. denitrificans* under controlled low-iron stress, it was necessary to take vigorous steps to remove iron present as a contaminant in reagent-grade chemicals. For a 12-liter batch, 70.8 g of succinic acid, 48.0 g of KH_2PO_4, 60.6 g of NaH_2PO_4, and 19.2 g of NH_4Cl were dissolved in 10 liters of glass-distilled deionized water. This solution, pH ca. 4.5, was autoclaved and allowed to sit at 4°C for 3 to 4 days to allow iron salts to coagulate. The solution was filtered through a 0.2-μm membrane and, after adjustment of the pH to 7.0, was passed through a column of 1,500 g of Chelex-100 resin (Bio-Rad Laboratories); the pH of this eluate was also adjusted to 7.0. Since this treatment also removed divalent cations and other multivalent elements in addition to iron, these components were added as a supplement following the Chelex column so that complete minimal salts medium contains the following divalent cations and trace elements: $MgSO_4$ (1.7 mM), Ca^{2+} (182 μM), Mn^{2+} (10 μM), Zn^{2+} (1 μM), Cu^{2+} (0.1 μM), and Co^{2+} (0.01 μM). Finally, 300 ml of Chelex-treated 20% Tween 80 solution was added to prevent clumping of cells, and the final volume was adjusted to 12 liters with distilled deionized water. The degree of low-iron stress could then be carefully controlled by addition of a known quantity of ferric nitrilotriacetate to this minimal salts medium.

P. denitrificans ATCC 17741 was obtained by rehydration of lyophilized cultures directly derived from the ATCC culture and maintained on tryptic agar plates for no longer than 2 weeks. Individual colonies were inoculated into 20 ml of tryptic soy broth and incubated with rotary shaking for 18 to 24 h at 30°C. A 50-ml portion of minimal salts medium containing 1 μM Fe(III) was inoculated with 300 μl of the tryptic soy broth culture and then incubated with shaking at 30°C for 16 to 20 h. This culture was then used to inoculate a 250-ml culture flask of 50 ml of minimal salts plus 0.5 μM Fe(III) (low iron) or 50 ml of minimal salts plus 50 μM Fe(III) (high iron) to give a starting optical density at 660 nm (OD_{660}) of 0.030 to 0.035. The flask was incubated with shaking (120 rpm) at 30°C. Samples were withdrawn from these cultures at various times thereafter, generally beginning about 10 h after inoculation.

Cell growth was estimated from the turbidometric OD_{660}. Under our conditions, a cell suspension with an OD_{660} of 1.000 contained 0.25 mg of protein ml^{-1} and corresponded to 1.52 mg (wet weight) ml^{-1}. Catecholamide siderophore production was estimated by addition of 20 μl of 50 mM ferric nitrilotriacetate to 1.00 ml of culture sample, filtration through a 0.2-μm membrane, and spectrophotometric assay of the filtrate. Ferric catecholamide content was calculated by using the extinction coefficient for ferric parabactin ($\varepsilon_{516nm} = 3.1 \times 10^3\ M^{-1}\ cm^{-1}$ [6]).

An extract containing cellular polyamines was prepared by centrifugation of a volume of culture containing 10 OD_{660}

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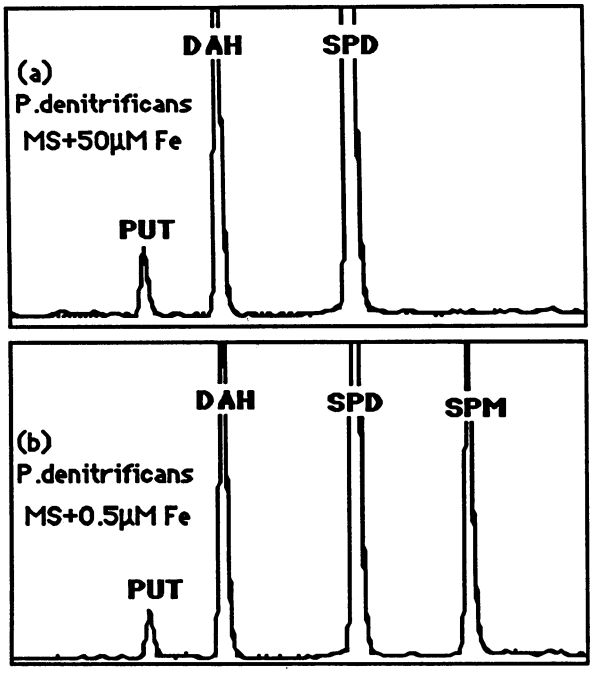

FIG. 1. HPLC of dansylated polyamines in 0.6 M $HClO_4$ extracts of *P. denitrificans* cultured aerobically (a) in an ammonium-succinate minimal salts medium supplemented with 50 μM ferric nitrilotriacetate (i.e., MS+50μM Fe) or (b) under conditions of iron deprivation (MS+0.5μM Fe) 1,6-Diaminohexane (DAH) was added to each sample as an internal standard. Each batch of samples processed also included samples containing PUT, SPD, and SPM at three concentrations as external standards.

units, suspension of the pellet in 500 μl of fresh minimal salts medium, and then addition of 500 μl of 1.2 M perchloric acid with vigorous mixing. This suspension was then subjected to three freeze-thaw cycles, using liquid nitrogen. Polyamines were analyzed by high-performance liquid chromatography (HPLC) of the dansyl derivatives on a C_{18} silica column eluted with an acetonitrile-water-methanol gradient. 1,6-Diaminohexane was added to each sample as an internal standard. Each batch of samples processed also included reference samples containing PUT, SPD, and SPM at three concentrations as external standards. We have reported a detailed description of the HPLC assay elsewhere (5).

The identification of SPM in iron-deprived *P. denitrificans* was confirmed by mass spectrometry. We collected and combined the presumed tetradansylspermine peaks from 48 analytical HPLC runs and removed the solvent by evaporation in a vacuum centrifuge (Savant Instruments). Fast atom bombardment mass spectra confirmed the molecular mass. Fragmentation "fingerprint" detail was obtained from chemical ionization mass spectra of the underivatized tetramine. An $HClO_4$ extract of iron-deprived *P. denitrificans* (ca. 5 g, wet weight) was fractionated by ion-exchange chromatography on AG50W-X8(H^+) (Bio-Rad Laboratories) to isolate the tetraamine(s) by the method of Sindhu and Cohen (40).

RESULTS

Figure 1 shows HPLC chromatograms of dansylated extracts of iron-sufficient and iron-deprived cultures together with an internal standard, 1,6-diaminohexane. Only two polyamines, PUT and SPD, are present in iron-replete cells

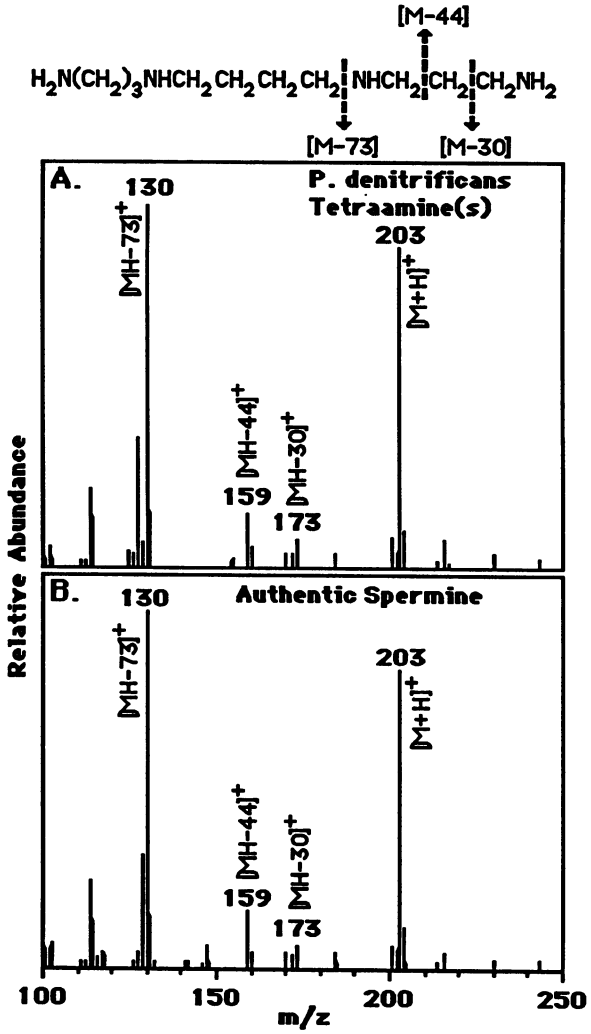

FIG. 2. Fractionation of an $HClO_4$ extract of iron-deprived *P. denitrificans* (ca. 5 g, wet weight) by ion-exchange chromatography on Bio-Rad AG50W-X8(H^+) to isolate the tetraamine(s) by the method of Sindhu and Cohen (40). Chemical ionization mass spectrometry of the natural product (A) produced a fragmentation fingerprint essentially identical to that of an authentic sample of SPM (B), demonstrating SPM to be the only tetraamine produced by iron-deprived *P. denitrificans*.

(Fig. 1a), and SPM is below detection limits of the assay (<0.01 μmol g [wet weight]$^{-1}$). This finding is a chemotaxonomic characteristic (2, 9, 46) of *P. denitrificans* as a member of the α-3 subdivision of the eubacterial phylum *Proteobacteria* (formerly purple photosynthetic bacteria). However, low-iron stress results in the production of substantial amounts of SPM so that it becomes the second most abundant polyamine (Fig. 1b). Bacteria are known to produce tetramines which differ from SPM [i.e., $H_2N(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$] in the number of carbons in (i) the two alkyl segments that separate the primary amino nitrogens from the internal secondary amino nitrogens, and/or (ii) the alkyl segment that separates the internal secondary amino nitrogens from each other. Thus, combinations and permutations of three- and four-carbon segments are known: thermine (norspermine), $C_3C_3C_3$; spermine,

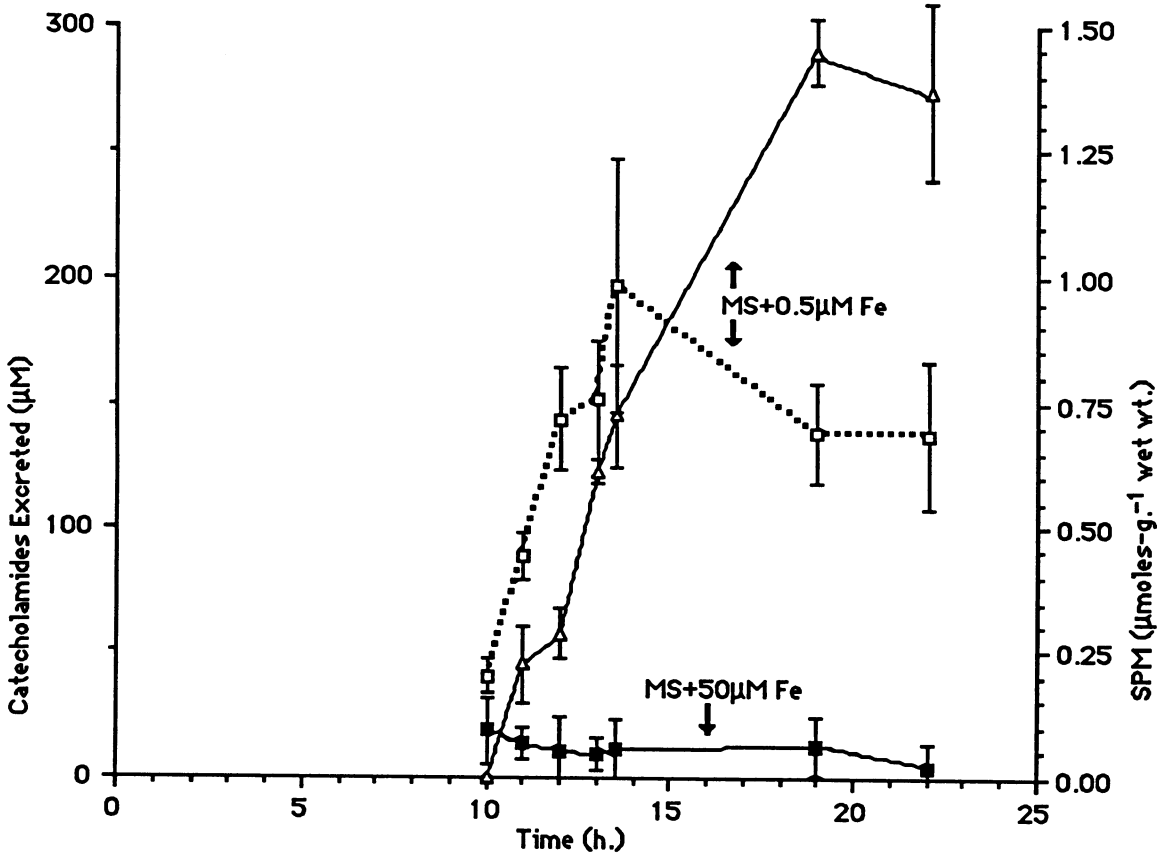

FIG. 3. Close temporal relationship between excretion of catecholamide siderophores (△) and increase in cellular SPM content (□) in iron-deprived cultures of *P. denitrificans*. Data indicate the SPM content (■) of cultures under identical conditions except that the medium was supplemented with 50 μM iron(III). These high-iron cultures did not excrete catecholamides (<10 μM at all time points; data not plotted). Data are presented as means with standard deviations (bars); n = 3 to 6. These combined data represent seven different experiments (i.e., conducted on different days over a span of 4 months). We have reported a detailed description of the preparation of low-iron minimal salts medium, assay methods for estimation of catecholamides, and specific culture conditions elsewhere (14). All experiments were inoculated to an OD_{660} of 0.030 to 0.034 at time zero. Growth rates for iron-deprived (MS+0.5μM Fe) and high-iron (MS+50μM Fe) cultures were almost identical, with doubling times of 1.87 and 1.99 h, respectively, although logarithmic growth plateaus at a lower final turbidity (OD_{660} of 3.5 to 4.5 at 14 to 16 h) in iron-depleted as compared with high-iron (final OD_{660} of 5 to 6) cultures.

$C_3C_4C_3$; thermospermine, $C_4C_3C_3$; canavalamine, $C_4C_3C_4$; and homospermine, $C_4C_4C_4$ (10, 20).

As dansylated synthetic samples of norspermine, SPM, and homospermine have very similar retention times in our HPLC system, we obtained mass spectral proof of the structure of the bacterial product. We collected and combined the presumed tetradansylspermine peaks from 48 analytical HPLC runs and removed the solvent in vacuo. Fast atom bombardment mass spectra of the residue confirmed it to be a tetradansyl derivative with the expected molecular mass of 1,135 Da (M + 1), indicating the tetraamine to be either SPM or thermospermine. The fast atom bombardment-mass spectrometry method, however, did not provide enough fragmentation detail to choose between these two possibilities. The chemical ionization mass spectrum of the underivatized tetraamine isolated from *P. denitrificans* (Fig. 2) proved much more informative and was identical to a chemical ionization mass spectrum of an authentic sample of SPM.

While small amounts of SPM are often detected in iron-sufficient (i.e., +50 μM Fe) cultures, the quantity is always substantially less than found in any culture producing detectable amounts (>10 μM) of catecholamide siderophores (i.e., phenotypically iron deprived). In contrast, iron deprivation had relatively modest effects on PUT and SPD. The following data are combined from seven different experimental batches over a period of several months: iron-deprived data (n = 26)—PUT, 0.61 ± 0.18; SPD, 4.68 ± 0.97; SPM, 0.72 ± 0.23 μmol g (wet weight)$^{-1}$ ± standard deviation; and iron-sufficient data (n = 26)—PUT, 0.66 ± 0.32; SPD, 6.82 ± 1.47; SPM, 0.06 ± 0.03 μmol g (wet weight^{-1} ± standard deviation.

Changes in polyamine content are known to occur in cells growing at different rates (8, 11, 40), but under our culture conditions *P. denitrificans* grows at almost the same rate in media containing 50 μM iron(III) (doubling time, 1.87 h) as in media containing 0.5 μM iron(III) (doubling time, 1.99 h.). Clearly, this small difference in growth rate is insufficient to explain the difference in polyamine contents. The temporal course of the increase in SPM content of the iron-deprived culture is revealing. We know from substantial previous experience (6) that, at about 10 to 11 h postinoculation into minimal salts medium containing 0.5 μM Fe(III), *P. denitrificans* begins to experience iron deprivation and responds over the course of the next several hours by (i) synthesis and excretion of the catecholamide siderophore L-parabactin (4,

6, 44); (ii) massive replacement of outer membrane components with several low-iron regulated proteins which eventually constitute ca. 60% of total outer membrane protein (26, 33, 45), including a high-affinity receptor for ferric L-parabactin which has been partially purified and characterized (7). These components constitute a stereospecific high-affinity transport system (K_m, 0.24 μM) for acquisition and transport of iron from the external milieu (6). Figure 3 demonstrates that the increase in SPM closely parallels, perhaps leading slightly, siderophore production. Note that the SPM of iron-replete cultures in the same phase of growth remains low throughout. To provide further support for a direct role of SPM in the response of *P. denitrificans* to low-iron stress, we conducted the reverse experiment. We reseeded cultures at the peak of the low-iron response (high SPM and siderophore production) into a high-iron (50 μM) minimal salts medium.

Figure 4 shows the effects of reseeding low-iron stressed cultures into a high-iron medium compared with reseeding into the same medium without supplemental iron. Cultures reseeded into fresh low-iron medium continue to excrete catecholamide siderophores at a rapid rate and maintain their high SPM (Fig. 4A and B, dashed lines). In contrast, in the presence of 50 μM iron(III), there is a rapid shutdown of siderophore production and a steep decline in SPM (Fig. 4A and B, solid lines). While SPM 30 min after reseed is only slightly lower than that of the original inoculum, there is a steep decline thereafter. Five hours (2.5 generations) after reseed, SPM is nearly below detection limits (≤0.01 μmol g [wet weight]$^{-1}$). This corresponds to more than a 50-fold decrease in SPM content and must represent an active metabolic disposition of SPM, in contrast to the 6-fold decrease calculated for passive dilution of the original content in 2.5 generations.

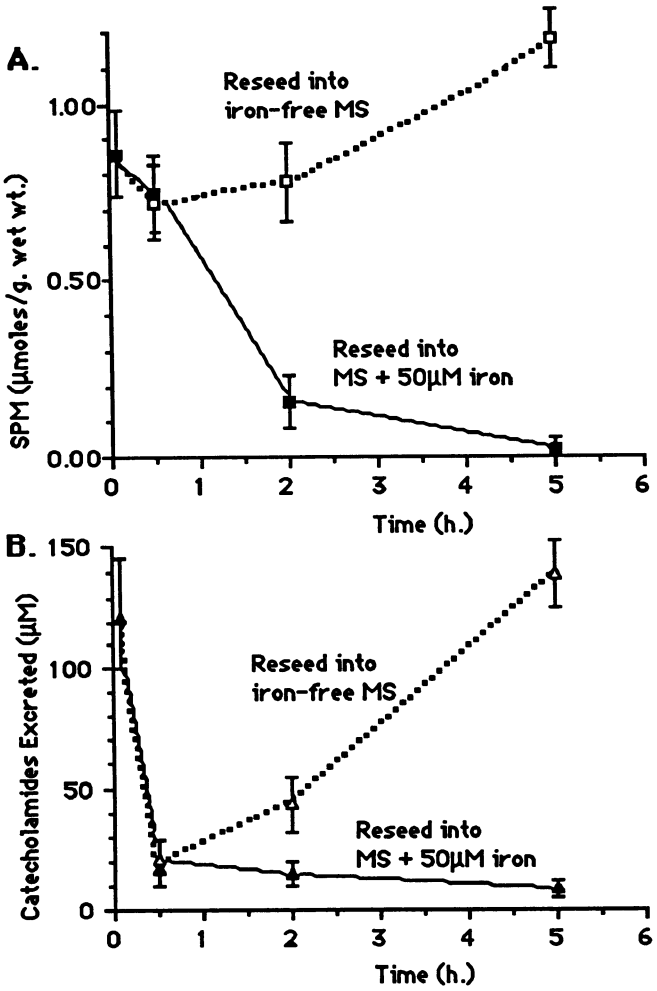

FIG. 4. Reversibility of the effects of iron deprivation. Briefly, an iron-deprived culture (13-h growth in minimal salts plus 0.5 μM Fe; see Fig. 3) was centrifuged at 25°C. The pellet was resuspended to an OD of 0.58 in 50 ml of fresh minimal salts medium without supplemental iron (□, △) or with 50 μM iron(III) added (■, ▲) and incubated in a 30°C water bath with shaking (120 rpm). Data are presented as means ± standard deviations of three separate experiments. Prior to reseeding, the cultures contained 0.86 ± 0.12 μmol of SPM g (wet weight)$^{-1}$ and were secreting catecholamides (130 ± 15 μM in the external medium) as represented by the time zero data. Samples of cultures taken 30 min after reseeding contained slightly decreased amounts of PUT, SPD, and SPM compared with the parent culture. No differences were apparent between iron-supplemented and iron-free reseed cultures at this time. However, 2 h after reseeding, the iron-supplemented cultures showed a marked decline in SPM. SPM was at or below limits of detection (<0.010 μmol g [wet weight]$^{-1}$) 5 h after reseeding into iron-supplemented medium. A small amount (21 ± 3 μM) of ferric siderophore was detected in the external medium of the iron-supplemented reseeds at 30 min, with decreasing amounts at later time points. This probably represents some residual carryover present in the original inoculum as well as some slight additional excretion during the initial 30 min after iron supplementation. In contrast, the iron-free reseed cultures maintained high SPM levels present prior to reseeding and continued to excrete substantial amounts of catecholamides.

DISCUSSION

These results suggest a specific role for SPM in the response of *P. denitrificans* to low-iron stress. There is no evidence that SPM is a precursor in the biosynthesis of catecholamide siderophore production. Parabactin has a triamine backbone which is donated by SPD directly. Unlike [^{14}C]SPD, [^{14}C]SPM was not a substrate for the in vitro parabactin-synthesizing system isolated from *P. denitrificans* by Tait (43).

The protein-synthesizing machinery of extreme thermophiles exhibits an absolute requirement for tetramines (16). Polyamines assist in the formation of stable rRNA, stimulate aminoacylation of tRNA, and improve the fidelity of translation of the nascent polypeptide. In mesophilic bacteria, these processes do not exhibit an absolute requirement for a tetramine, although the tetraamines are the most effective of the polyamines (40, 42). In many systems an order of magnitude separates each of the three polyamines from the next with an order of SPM > SPD > PUT (1, 16, 42). Thus, SPM may function at the level of translation.

SPM can bind to DNA in a sequence-specific manner (14, 15, 28) and is known to influence DNA superhelicity (3, 29, 30, 32). Several reports suggest a role for DNA superhelicity in the expression of genes associated with microbial iron transport (13, 36). It is not known whether these bacteria also produce large amounts of SPM when deprived of iron as does *P. denitrificans*, but these reports relating DNA tertiary structure and expression of low-iron regulated genes are consistent with the hypothesis that *P. denitrificans* may produce SPM in response to iron deprivation to stabilize DNA structure that most favors transcription of the low-iron regulated genes. The potential relationships of SPM and iron transport in other eubacteria is a subject of ongoing investigations in our laboratory. Whether or not there are phylo-

genetic trends of general significance in microbial evolution, these observations underscore the need for a careful definition of culture conditions including iron content of the medium in chemotaxonomic studies.

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