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Outer membrane proteins induced upon iron deprivation of *Paracoccus denitrificans*

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Deprivation of two strains of *Paracoccus denitrificans* of iron elicited substantial siderophore production, and the associated cell envelope protein changes were examined. In strain DSM 413 four new outer membrane proteins in the M_r range 84000 to 76000 were induced, and an M_r 23000 protein disappeared, upon iron deprivation. In strain DSM 65 three new outer membrane proteins in the M_r range 81000 to 76000 were induced.

Over a number of years we have been interested in the cell envelope of the Gram-negative bacterium *Paracoccus denitrificans*, which shows some unusual properties in being highly permeable to lysozyme and hydrophobic agents [1–3]. We have recently isolated the outer membrane from the organism and examined its protein composition [4]. One of our goals is to further characterize and identify *P. denitrificans* cell envelope proteins. In a variety of bacterial species, coincident with iron deprivation and siderophore production is the production of high molecular weight outer membrane proteins, believed to be receptors functioning in the uptake of ferric ion-siderophore complexes [5]. *P. denitrificans* has been shown to produce catechol-type siderophores when grown in media deficient in iron [6,7]. Here we report that deprivation of the organism of iron induces the synthesis of three or four high-molecular weight outer membrane proteins, not produced in iron-sufficient cultures.

P. denitrificans strains DSM 65 (= ATCC 17741) and DSM 413 (= ATCC 19367 = NCIB 8944) [8] were used. For iron deprivation experiments the organisms were grown in succinate-salts medium [9] with different concentrations of ferric citrate, and with sodium molybdate omitted [10]. All cultures were grown with shaking (200 rpm) at 30°C. A starter culture was obtained by inoculating 100 ml medium, containing 20 μM ferric citrate, in a 250 ml Erlenmeyer flask with one loop of culture from a slant and incubating for 24 h. Starter culture (5 or 20 ml) was used to inoculate two 2-l Erlenmeyer flasks each containing 500 ml medium (0.8 μM ferric citrate), which were incubated until they reached an $A_{625\ nm}$ of 0.2–0.5 (pre-switch growth). Then the cultures were harvested in sterile centrifuge bottles in a rotor operated for 5 min at room temperature and 9880 $\times g$ ($r_{av.}$ 8.7 cm), and resuspended and pooled in pre-warmed (30°) medium containing no ferric citrate (20 ml). This suspension was equally divided between two flasks of medium (500 ml/2-l Erlenmeyer flask) containing 0 or 0.8 μM ferric citrate (post-switch growth). An iron-sufficient cul-

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Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

ture was obtained by pre- and post-switch growth in 100 μM ferric citrate. The post-switch cultures were incubated and samples were taken at intervals for determination of culture turbidity, siderophore production and cell envelope preparation.

In order to examine the kinetics of siderophore production and cell envelope protein changes, samples were taken 4 h (100 ml sample), 8 h (60 ml sample), 12 h and 24 h (30 ml samples) during post-switch growth. Whole cells were harvested by centrifuging in a rotor operated for 10 min at 4°C and $1600 \times g$ ($r_{av.}$ 8.95 cm). Catechol production in culture supernatants was determined using the Arnow reagent as described by Corbin and Bulen [11], with 2,3-dihydroxybenzoic acid as standard Lysozyme-cell envelopes were obtained by a procedure based on that of Witholt et al. [12]. After lysis of osmotically sensitive spheroplasts the suspensions were centrifuged in a rotor operated for 30 min at 4°C and $2600 \times g$ ($r_{av.}$ 8.95 cm) to remove whole cells and poly-β-hydroxybutyrate granules. Lysozyme-cell envelopes were collected by centrifugation in a rotor operated for 60 min at 4°C and $240\,000 \times g$ ($r_{av.}$ 8.12 cm) and washed by resuspension in cold distilled water followed by centrifugation. Lysozyme-cell envelopes were resuspended in distilled water and stored at −15°C.

To isolate cytoplasmic and outer membranes post-switch cultures were incubated for 12 h before the entire culture (500 ml) was harvested and cells were broken by passage through a French pressure cell. After removal of whole cells and poly-β-hydroxybutyrate granules as described above, cell envelopes were obtained by centrifugation in a rotor operated for 60 min at 4°C and $240\,000 \times g$ ($r_{av.}$ 8.12 cm). Cytoplasmic and outer membranes were separated by sucrose density gradient centrifugation by a method (Ref. 4 and Nauyalis, P.A., Hindahl, M.S. and Wilkinson, B.J., unpublished data) based upon those of Schnaitman [13] and Koplow and Goldfine [14]. Cell envelopes were loaded on gradients of 6 ml 2.25 M, 10 ml 1.44 M and 16 ml 0.77 M sucrose in 0.01 M Hepes buffer (pH 7.4), which were centrifuged in an SW 28.1 rotor (Beckman Instruments, Palo Alto, CA, U.S.A.) operated for 20–24 h at 4°C and $90\,000 \times g$ ($r_{av.}$ 12.2 cm). A simple pattern of two bands was obtained, the upper and lower of which were identified as cytoplasmic and outer membranes, respectively, on the basis of density, assay of marker molecules and enzymes, and protein composition (Ref. 4 and Nauyalis et al., unpublished data). Cell envelopes, cytoplasmic and outer membranes were suspended in distilled water and stored at −15°C. Protein was estimated by the method of Lowry et al. [15] using bovine serum albumin, Fraction V (Sigma Chemical Co., St. Louis, MO, U.S.A.) as standard.

Cell envelope and membrane proteins were examined by sodium dodecylsulfate(SDS)-polyacrylamide gel electrophoresis according to Lugtenberg et al. [16] using a 3.5 cm 4.6% total, 2.6% cross-linking acrylamide stacking gel and a 10.5 cm 12.2% total, 1.8% cross-linking acrylamide separating gel. The gels were run at constant current (30 mA) until the dye front reached the bottom of the gel and were stained with Coomassie blue R250.

In an experiment where the pre-switch ferric citrate concentration was 20 μM, only small amounts of siderophore were produced after 24 h of post-switch iron deprivation (data not shown). Significant siderophore production occurred when the pre-switch ferric citrate concentration was 0.8 μM. This and growth of strain DSM 413 are shown in Fig. 1. Similar results were obtained with strain DSM 65 (data not shown). Deprivation of iron caused only very minor reductions in the growth rates and final turbidities achieved. However, the cell pellet under iron-sufficient conditions (100 μM ferric citrate) remained a salmon pink color, whereas by 8 h the cell pellets from the iron-deficient cultures were whitish. This may have been due to limitation of cytochrome formation under these conditions. Also, the iron-sufficient culture retained a faint pink coloration throughout the experimental period, whereas the iron-deficient cultures often turned dirty yellowish after 12 h and deep burgundy red after 24 h. It is not known whether these color changes are due to siderophore production. Tait [6] reported that iron-deficient cultures were yellow and turned red upon addition of ferric citrate.

Colorimetric assays revealed marked siderophore production by 8 h post-switch growth in 0.8 μM ferric citrate and in medium without ferric citrate. Siderophore production generally occurred somewhat more rapidly and to a somewhat greater

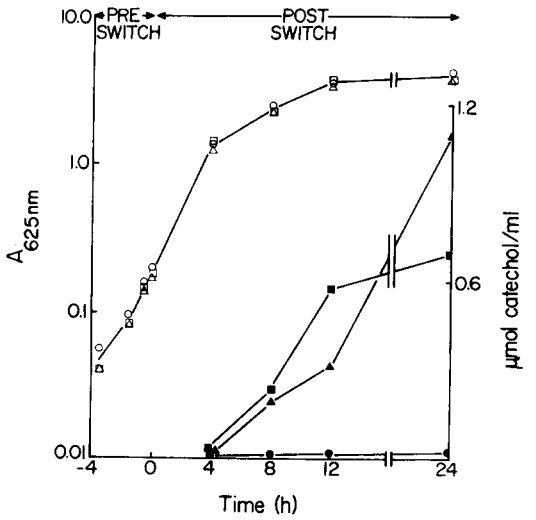

Fig. 1. Growth and siderophore production by *P. denitrificans* strain DSM 413 under conditions of iron sufficiency and deprivation. Samples were removed at intervals for the determination of turbidity (open symbols) and estimation of catechol in culture supernatants (filled symbols); ○ ●, 100 µM ferric citrate present pre- and post-switch; △ ▲, 0.8 µM ferric citrate present pre- and post-switch; □ ■, 0.8 and 0 µM ferric citrate present pre- and post-switch, respectively.

extent in medium containing no ferric citrate. The amount of siderophore produced showed some variation between experiments (0.16–0.33 µmol/ml culture in five experiments, 0.8 µM ferric citrate, 12 h post-switch, strain DSM 65; 0.14 and 0.32 µmol/ml culture in two experiments, 0.8 µM ferric citrate, 12 h post-switch, strain DSM 413). The colorimetric assays indicated that low levels of siderophore were produced in the iron-sufficient culture. It is not known whether this represents a background level of true siderophore production, or whether it is an artifact such as an unrelated substance released into the medium giving a low color yield.

Samples were taken at intervals during post-switch growth and lysozyme-cell envelopes were prepared by osmotic lysis of spheroplasts produced by lysozyme-EDTA treatment. This method of cell envelope preparation was convenient for their preparation from a relatively large number of samples (typically 12). In both strains additional proteins were produced in iron-deprived cells. The results of SDS-polyacrylamide gel electrophoresis of the lysozyme-cell envelopes from strain DSM 413 are shown in Fig. 2. In strain DSM 413 three new proteins in the M_r range 83000 to 74000 were observed in lysozyme-cell envelopes prepared from cells deprived of iron for 4 h. In the equivalent samples of strain DSM 65 three new proteins in the M_r range 82000 to 79000 were observed. In samples taken at later times from iron-deprived strain DSM 413 cells the M_r 74000 protein was not detected but an M_r 72000 and a faint 70000 protein were detected. Production of the new proteins was not due to deprivation of citrate because they were produced in cultures deprived of iron, but that contained citrate. In both strains increased amounts of new proteins relative to total lysozyme-cell envelope protein were present in samples taken at later times. Induction of new lysozyme-cell envelope proteins was detected after 4 h of iron deprivation, whereas only small amounts of siderophore had been produced. This suggests that the effects of iron deprivation were more sensitively detected, by SDS-polyacrylamide gel electrophoresis, as new cell envelope proteins than by the colorimetric assay for siderophore production.

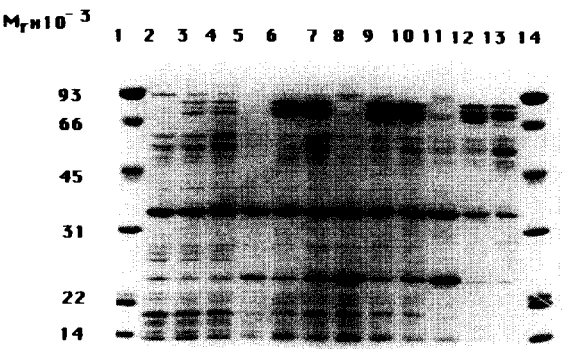

Fig. 2. Cell envelope protein changes in *P. denitrificans* strain DSM 413 upon iron deprivation. Experimental conditions were as in Fig. 1 and lysozyme-cell envelopes were prepared and examined by SDS-polyacrylamide gel electrophoresis. Lanes 1 and 14 5 µg proteins used as M_r standards: phosphorylase B (92500), bovine serum albumin (66200), ovalbumin (45000), carbonic anhydrase (31000), soybean trypsin inhibitor (21500), and lysozyme (14400); lanes 2, 5, 8 and 11, 100 µM ferric citrate; lanes 3, 6, 9 and 12 0.8 µM ferric citrate; lanes 4, 7, 10 and 13 no ferric citrate. Lanes 2–4, 4 h samples; 5–7, 8 h samples; 8–10, 12 h samples; 11–13, 24 h samples.

In addition, deprivation of iron led to the disappearance of a protein of M_r 23000 (Fig. 2). Further work in this laboratory has shown that the production of this protein is dependent on the addition of iron to the medium and the growth phase of the cells (Wee, S., Madiraju, M. and Wilkinson, B.J., unpublished data).

In order to determine the localization of the iron deprivation-induced proteins, cell envelopes were prepared using the French pressure cell, and were separated into cytoplasmic and outer membranes by sucrose density gradient centrifugation. The fractions were examined by SDS-polyacrylamide gel electrophoresis and results obtained with strain DSM 413 are shown in Fig. 3. The new proteins were present in the outer membrane in both strains. Four new proteins were found in strain DSM 413 (M_r range 84000 to 76000) and three new proteins in strain DSM 65 (M_r range 81000 to 76000), when both strains were grown in 0.8 μM ferric citrate post-switch. Compared to lysozyme-cell envelopes it appeared that new proteins ran with slightly different molecular weights in the two preparations. No changes were noted in the proteins of cytoplasmic membrane on comparison of iron-sufficient and -deficient cultures.

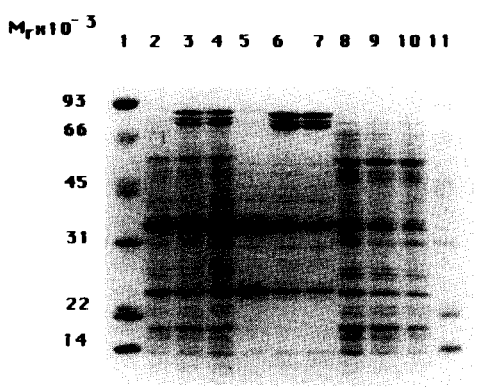

Fig. 3. Outer membrane localization of iron deprivation-induced proteins. Cell envelopes were prepared from strain DSM 413 using the French pressure cell from 12 h post-switch cultures with 100, 0.8 and 0 μM ferric citrate, and were separated into cytoplasmic and outer membranes by sucrose density gradient and examined by SDS-polyacrylamide gel electrophoresis. Lanes 1 and 11, standard proteins; lanes 2, 3 and 4, cell envelopes, 100 0.8 and 0 μM ferric citrate, respectively; lanes 5, 6 and 7, outer membranes, 100, 0.8 and 0 μM ferric citrate, respectively; lanes 8, 9 and 10, cytoplasmic membranes, 100, 0.8 and 0 μM ferric citrate, respectively.

The induction of membrane proteins in response to iron deprivation appears to be a common theme in Gram-negative bacteria [5]. Typically iron deprivation induces the synthesis of outer membrane proteins in the M_r range 70000–90000, and these proteins are believed to function as receptors for siderophores [5,17,18]. *Agrobacterium tumefaciens* excretes a siderophore called agrobactin upon iron deprivation [5], which is structurally very similar to one of the *P. denitrificans* siderophores, parabactin [6,7]. Concomitant with the production of agrobactin, at least three new proteins with M_r of approximately 80000 have been observed in the *A. tumefaciens* cell envelope [19].

Iron induced proteins in the *P. denitrificans* cell envelope were similar in molecular weight to those reported for *A. tumefaciens*, and for other Gram-negative bacteria. These proteins were shown to be present in the outer membrane, and did not appear to be produced under conditions of iron sufficiency. This report provides the first identification of any *P. denitrificans* outer membrane proteins, and suggests that their function may be as siderophore receptors.

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References

1 Scholes, P.B. and Smith, L.C. (1968) Biochim. Biophys. Acta 153, 350–362
2 Wilkinson, B.J. (1977) FEMS Microbiol. Lett. 2, 285–288
3 Hindahl, M.S., Wee, S., Banks, D.H., Tsang, J.C. and Wilkinson, B.J. (1981) Arch. Microbiol. 130, 307–311
4 Nauyalis, P.F. and Wilkinson, B.J. (1983) Abstr. Annu. Mtg. Am. Soc. Microbiol. K200, p. 210
5 Neilands, J.B. (1982) Annu. Rev. Microbiol. 36, 285–309
6 Tait, G.H. (1975) Biochem. J. 14, 191–204
7 Peterson, T. and Neilands, J.B. (1979) Tetrahedron Lett. 50, 4805–4808
8 Nokhal, T.H. and Mayer, F. (1979) Antonie van Leeuwenhoek 45, 185–197
9 Chang, J. and Morris, J.G. (1962) J. Gen. Microbiol. 29, 301–310
10 Page, W.J. and von Tigerstrom, M. (1982) J. Bacteriol. 151, 237–242
11 Corbin, J.L. and Bulen, W.A. (1969) Biochemistry 8, 757–762
12 Witholt, B., Bockhart, M., Brock, M., Kingma, J., van

Heehuizen, H. and de Ley, L. (1976) Anal. Biochem. 74, 160–170
13 Schnaitman, C.A. (1970) J. Bacteriol. 104, 890–901
14 Koplow, J. and Goldfine, H. (1974) J. Bacteriol. 117, 527–543
15 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
16 Lugtenberg, B., Meijers, J., Peters, R., Van de Hock, P. and Alphen, L.V. (1975) FEBS Lett. 58, 254–258
17 Klebba, P.E., McIntosh, M.A. and Neilands, J.B. (1982) J. Bacteriol. 149, 880–888
18 Braun, V., Hancock, R.E.W., Hantke, K. and Hartmann, A. (1976) J. Supramol. Struct. 5, 37–58
19 Leong, S.A. and Neilands, J.B. (1981) J. Bacteriol. 147, 482–491