Environmental Microbiology (2003) doi: 10.1046/j.1462-2920.2003.00465.x

Proteome reference map of *Pseudomonas putida* strain KT2440 for genome expression profiling: distinct responses of KT2440 and *Pseudomonas aeruginosa* strain PAO1 to iron deprivation and a new form of superoxide dismutase

S. Heim,* M. Ferrer, H. Heuer, D. Regenhardt, M. Nimtz and K. N. Timmis
Department of Environmental Microbiology, GBF – German Research Centre for Biotechnology, Mascheroder Weg 1, D-38124 Braunschweig, Germany.

Summary

The genome sequence of *Pseudomonas putida* strain KT2440, a nutritionally versatile, saprophytic and plant root-colonizing Gram-negative soil bacterium, was recently determined by K. E. Nelson *et al*. (2002, *Environ Microbiol* 4: 799–808). Here, we present a two-dimensional gel protein reference map of KT2440 cells grown in mineral salts medium with glucose as carbon source. Proteins were identified by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) analysis, in conjunction with an in-house database developed from the genome sequence of KT2440, and ≈ 200 two-dimensional gel spots were assigned. The map was used to assess the genomic response of KT2440 to iron limitation stress and to compare this response with that of the closely related facultative human pathogen *Pseudomonas aeruginosa* strain PAO1. The synthesis of about 25 proteins was affected in both strains, including four prominent upregulated ferric uptake regulator (Fur) protein-dependent proteins, but there were also striking differences in their proteome responses, for example in the expression of superoxide dismutases (Sod), which may indicate important roles of iron-responsive functions in the adaptation of these two bacteria to different lifestyles. The Sod enzyme of KT2440 was shown to be a novel heterodimer of the SodA and SodB polypeptides.

Received 12 December, 2003; revised 27 March, 2003; accepted 28 March, 2003. *For correspondence. E-mail she@gbf.de; Tel. (+49) 531 6181 557; Fax (+49) 531 6181 144.

Introduction

Bacteria of the species *Pseudomonas putida* are ubiquitous, metabolically versatile, Gram-negative, saprophytic soil and water bacteria that are nutritional opportunists par excellence, which are assumed to play a significant role in the recycling of organic wastes in the environment and, hence, in the maintenance of environmental quality (see Timmis, 2002). Strain KT2440 (Bagdasarian *et al*., 1981; Regenhardt *et al*., 2002) is a paradigm of the species *P. putida* and is used worldwide as a workhorse for genetic and physiological studies (e.g. Cases *et al*., 2001; Kasai *et al*., 2001; Llamas *et al*., 2003) and for the development of biotechnological applications (e.g. Klinke *et al*., 2000; Ronchel and Ramos, 2001; Bühler *et al*., 2002); it is also a certified biosafety strain (Bagdasarian *et al*., 1981; Federal Register, 1982) for recombinant DNA experiments and applications. The recent determination of its genome sequence (Nelson *et al*., 2002) provides a new framework for the elucidation of the genomic and physiological basis of the metabolic versatility and environmental success of this fascinating bacterium.

Investigation of responses of bacteria to environmental signals has until recently been hindered by a paucity of approaches to gain insights into global metabolic and regulatory networks. Development of proteomic tools in combination with complete genome sequences now offers the opportunity of relating genome-wide expression responses to environmental stimuli (Cordwell *et al*., 2001). In order to facilitate functional genomic analysis of KT2440, we have generated a first two-dimensional protein reference map and used it to display the genomic response of KT2440 to one important environmental signal, namely deprivation of iron, a key nutrient of microbes and essential cofactor of a number of respiratory enzymes of aerobic bacteria. We have compared this response with that of the closely related *Pseudomonas aeruginosa* strain PAO1, a model of facultative pathogens that can live as soil saprophytes, and have habitat spectra which overlap with those of *P. putida*.

Results

A two-dimensional protein reference map of P. putida *strain KT2440*

Two-dimensional gels of proteins extracted from cells of *P. putida* strain KT2440 grown under standard conditions, i.e. a low phosphate mineral salts medium with glucose as carbon source, were used to create a protein reference map. Figure 1 shows a silver-stained two-dimensional gel on which some 2356 protein spots were resolved. Some 200 well-resolved polypeptides visible in Coomassie-stained two-dimensional gels were identified by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) analysis. These polypeptides are indicated by numbers in Fig. 1 and listed in Table 1. Around 60% represent abundant proteins, whereas more than 30% belong to the class of low abundance proteins. Some 5% were hypothetical or conserved hypothetical proteins of unknown function.

In some cases (about five in this work), up- or downregulation in response to changes in growth conditions provided insights into the possible roles of proteins of unknown function. For example, the 45 kDa protein specified by Orf01685 (spot 6 in Fig. 1) was only expressed in low phosphate media. Sequence comparisons indicated that its N-terminus exhibited 80% similarity to that of the

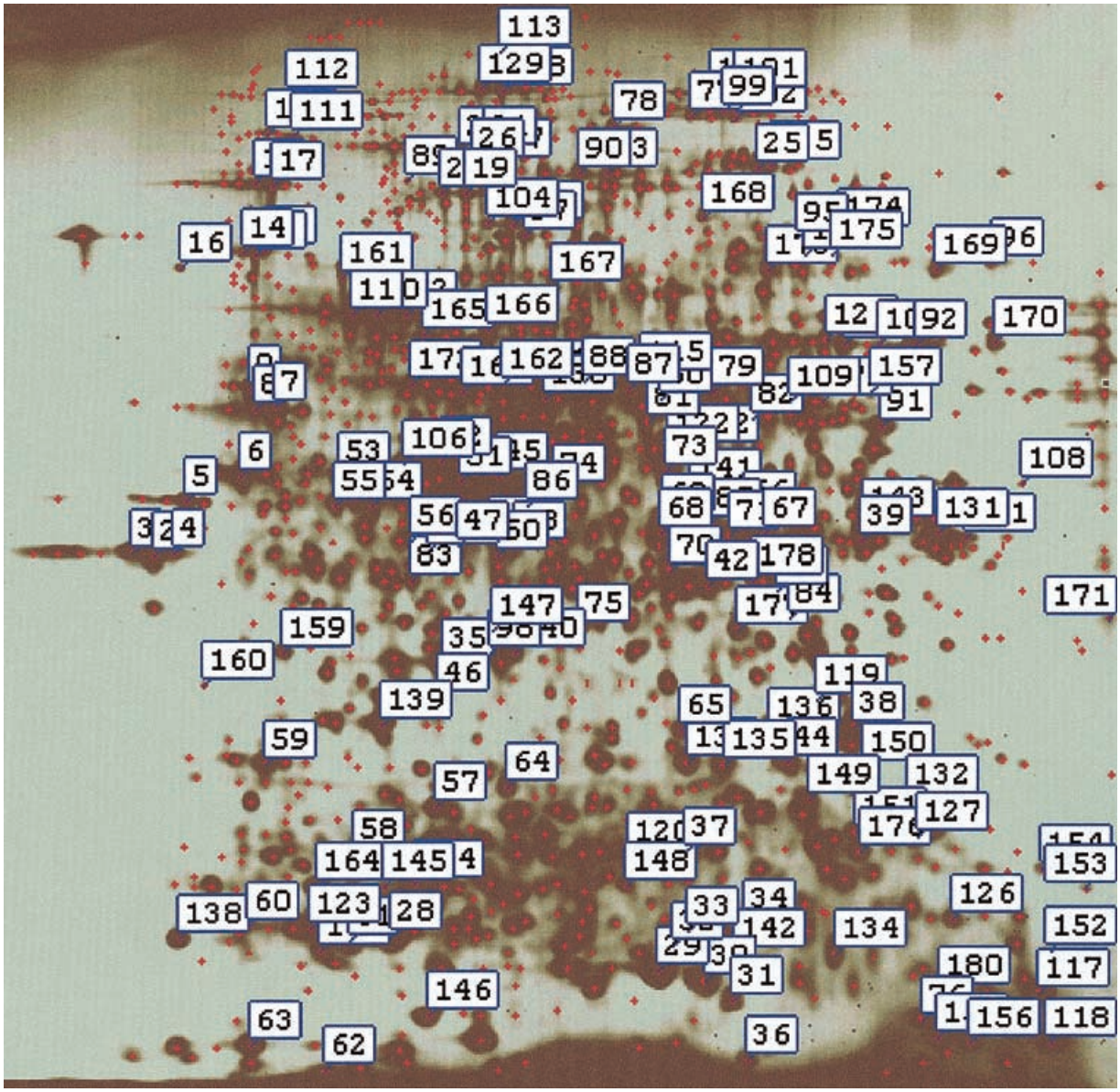

Fig. 1. Proteome reference map of *P. putida* strain KT2440. Silver-stained two-dimensional gel obtained from cells grown under standard conditions (see *Experimental procedures*). Information on the identified proteins can be found in Table 1 (see also *Supplementary material*).

Table 1. List of identified proteins of *P. putida* KT2440.

Spot number	Protein name	pI	MW (kDa)
1	Flagellin FliC	4.24	67
2	Outer membrane protein F	4.5	37
3	Outer membrane protein F	4.5	37
4	Outer membrane protein F	4.5	37
5	Outer membrane protein D	4.84	46
6	Hypothetical protein Orf01685	4.87	45.3
7	ATP synthase β-chain	4.73	49.3
8	ATP synthase β-chain	4.73	49.3
9	Trigger factor	4.8	48.5
10	Heat shock protein (Hsp60), GroEL protein	4.97	56.7
11	Heat shock protein (Hsp60), GroEL protein	4.97	56.7
12	Heat shock protein (Hsp60), GroEL protein	4.97	56.7
13	30S ribosomal protein S1 RspA	4.84	63.2
14	30S ribosomal protein S1 RspA	4.84	63.2
15	30S ribosomal protein S1 RspA	4.84	63.2
16	N utilization protein substance A	4.8	54.6
17	DnaK protein	4.69	68.8
18	DnaK protein	4.69	68.8
19	Polyribonucleotide nucleotidyltransferase Pnp	5.12	75
20	Polyribonucleotide nucleotidyltransferase Pnp	5.12	75
21	Translation elongation factor G FusA	5.25	78
22	Translation elongation factor G FusA	5.25	78
23	Probable solbitol dehydrogenase	5.5	81
24	Probable solbitol dehydrogenase	5.5	81
25	Catalase/peroxidase HPI	5.64	82
26	Outer membrane ferripyoverdine receptor FpvA	5.46	89.7
27	Outer membrane ferripyoverdine receptor FpvA	5.46	89.7
28	Peroxidase	5.06	21.7
29	Iron superoxide dismutase SodB	5.6	22
30	ATP synthase F1, delta chain AtpH	5.57	19
31	Conserved hypothetical protein Orf03678	5.64	18.5
32	ATP-dependent clp protease, proteolytic subunit	5.53	23.5
33	Hydrolase isochorismate family Orf07396	5.4	23
34	Transcriptional regulator TtgR	5.68	23
35	Elongation factor EF-Ts	4.99	31
36	Multicopy mutation suppressor DksA	5.6	17
37	2-Keto-3-deoxy-6-phosphogluconate aldolase AlkD	5.4	24.7
38	Succinyl-CoA-synthase alpha chain	5.89	30.1
39	Succinyl-CoA-synthase beta chain	5.8	41
40	Metallo-beta-lactamase family protein	5.41	37.7
41	Putrescine-binding protein PotF	5.68	38
42	Keto acid reductoisomerase IlvC	5.48	36.3
43	Ribosomal 5S rRNA E loop-binding protein Ctc/L25/TL5	6	23.2
44	5,10 Methylene tetrahydrofolate reductase	5.7	31
45	Elongation factor EF-Tu	5	43.5
46	Electron transfer flavoprotein, alpha subunit	5.07	31
47	Phosphoglycerate kinase	5.15	40
48	Binding component of ABC transporter	5.6	37.8
49	Keto acid dehydrogenase E1 beta subunit	5.3	40
50	Delta-aminolaevulinic acid dehydratase HemB	5.17	35.2
52	Malic enzyme	4.96	45
51	Translation elongation factor EF-Tu TuF	5	43.5
53	Enolase Eno	4.92	45.5
54	DNA-directed RNA polymerase alpha subunit RpoA	4.96	36.6
55	Cell division protein Ftsz	4.8	42
56	Syrp protein	4.99	36.9
57	Tryptophan synthase alpha chain	5.04	28.5
58	Amino acid ABC transporter, periplasmic binding protein Orf08103	5.22	27.9
59	Translation elongation factor P Efp	4.7	21.3
60	Inorganic pyrophosphatase Ppa	4.77	19
61	Alkyl hydroperoxide reductase AhpC small subunit	4.8	20
62	30S ribosomal protein S6	4.94	16.3
63	MenG	4.7	17.6
64	Conserved hypothetical protein Orf02515	5.4	30
65	Thiosulphate sulphur transferase	5.55	31
66	Branched-chain amino acid ABC transporter, amino acid-binding protein BraC	5.6	37
67	ABC sugar transporter, periplasmic sugar-binding protein	5.7	43
68	ABC sugar transporter, periplasmic sugar-binding protein	5.7	43
69	Branched-chain amino acid ABC transporter, amino acid-binding protein BraC	5.6	37

Table 1. *Cont.*

Spot number	Protein name	pI	MW (kDa)
70	GDP-mannose-4,6-dehydratase Gmd	5.5	38
71	Biotin biosynthetase BioB	5.5	39
72	N-carbamoyl-β-alanine amidohydrolase	6.2	50
73	Isocitrate dehydrogenase, NADP dependent Icd	5.4	45.6
74	Aminotransferase, class I	5.44	43.7
75	General amino acid ABC-transporter, periplasmic binding protein Aapj	5.8	36.4
76	Outer membrane protein H1	5.9	19.2
77	Outer membrane haem receptor	5.75	95
78	Clp protein ClpB-1	5.4	95
79	Arginine deiminase ArcA	5.5	46
80	Arginine deiminase ArcA	5.5	46
81	Agglutination protein TolC	5.4	5.53
82	Fumarate hydratase FumC	5.65	48.5
83	4-Hydroxyphenylpyruvate dioxygenase Hppd	4.92	40
84	Carbamate kinase	5.6	33
85	Branched-chain amino acid ABC transporter, periplasmic amino acid-binding protein	5.58	38
86	Glutaryl-CoA dehydrogenase GcdH	5.8	43
87	Isocitrate lyase AceA	5.34	49
88	ATP-synthase F1, alpha subunit AtpA	5.37	55
89	Fimbrial usher protein, putative	5	86
90	Outer membrane ferrisiderophore receptor	5.5	76
91	Serine hydroxymethyl transferase	5.85	45
92	2-Oxoglutarate dehydrogenase, lipoamide dehydrogenase component LpdG	5.93	49.9
93	Dihydrolipoamide dehydrogenase	5.93	49.9
94	Outer membrane copper receptor C OprC	5.86	74.5
95	Outer membrane copper receptor C OprC	5.86	74.5
96	Quinoprotein ethanol dehydrogenase	6.6	69
97	Peptidyl-prolyl cis-trans isomerase D PpiD	5.38	71.4
98	Shikimate 5-dehydrogenase AroE	5.23	29.3
99	Pyruvate dehydrogenase AceE	5.56	99.3
100	Aconitate hydratase I AcnA	5.8	102
101	Aconitate hydratase I AcnA	5.8	102
102	Outer membrane haem receptor	5.75	95
103	Heat shock protein HtpG	5.2	71.6
104	Heat shock protein HtpG	5.2	71.6
105	Methyl-accepting chemotaxis transducer for inorganic phosphate CtpL	5.12	70.3
106	Para-aminobenzoate synthase, component I PabB	5.13	49
107	CoA hydrolase/transferase family protein	5.72	74
108	4-Hydroxybenzoate hydroxylase PobA	6.1	46
109	Aspartate ammonium-lyase AspA	5.78	52.4
110	Outer membrane ferric siderophore receptor Orf07154	4.93	82
111	ATP-dependent protease	4.8	89
112	Organic solvent tolerance protein	4.96	106
113	Carbamoylphosphate synthase large subunit CarbB	5.17	117
114	Glycerine kinase GlpK	5.13	55
115	S-adenosyl-homocysteine hydrolase AhcY	5.35	52
116	Penicillin tolerance protein	4.97	34.5
117	Conserved hypothetical protein Orf00938	6.6	20.7
118	Heat shock protein IbpA	5.94	16.3
119	D-β-Hydroxybutyrate dehydrogenase BdhA	6.3	26.8
120	Enoyl-CoA hydratase/isomerase FadBlx	5.43	27.7
121	Malate:quinone oxidoreductase Mqo	6.11	59.9
122	3-Oxoacyl-[acyl-carrier-protein] synthase I FabB	5.43	43.2
123	IPP isomerase type I family protein	4.8	19.8
124	UTP-glucose-1-phosphate uridyltransferase GalU	5.46	30.9
125	Formate dehydrogenase subunit	5.98	85.6
126	Hypothetical protein Orf04737	5.99	17.2
127	Histidine ABC transporter, periplasmic binding protein	6.02	26.5
128	Aconitate hydratase 2 AcnB	5.19	94.2
129	Aconitate hydratase 2 AcnB	5.19	94.2
130	Dihydroorotate dehydrogenase family protein	5.25	46
131	Ornithine carbamoyltransferase ArgF	6.21	39.7
132	Transporter, putative Orf04758	6.81	46.9
133	Conserved hypothetical protein Orf00278	5.53	31.6
134	Enoyl CoA-hydratase/isomerase family protein	5.8	27.9
135	Acetylglutamate kinase ArgB	5.57	31.9
136	Succinyl CoA-synthase alpha chain SucD	5.89	30.1
137	Peptidyl-prolyl cis-trans isomerase SlyD	4.83	20.5
138	Outer membrane protein OprG	4.98	24

Table 1. *Cont.*

Spot number	Protein name	pI	MW (kDa)
139	Nicotinate-nucleotide pyrophosphorylase NadC	5.36	30.2
140	Polysaccharide deacetylase family protein	5.64	35
141	Rieske 2Fe-2S family protein Orf03448	5.5	39
142	Intracellular proteinase I PfpI	5.6	20.5
143	Ornithine carbamoyltransferase ArgF	6.21	39.7
144	Imidazole glycerophosphate synthase, cyclase subunit	5.1	27
145	Cytidylate kinase	5.1	24
146	Thiolperoxidase	5	17
147	Malate dehydrogenase	5.2	30
148	3-Isopropylmalate dehydrogenase, small subunit	5.3	24
149	Ribosomal protein L25	5.7	21
150	Cell division inhibitor MinD	5.3	24.7
151	Pyridoxine-5-phosphate oxidase	5.9	24
152	Disulphide oxidoreductase	6.6	23
153	Probable ATP-binding component of ABC transporter	6.5	26
154	Probable TonB-dependent receptor	6.9	27.7
155	Bacterioferritin co-migratory protein	5.3	18.5
156	Acetolactate synthase isoenzyme III small subunit	5.9	17.7
157	Glu-t-RNA amidotransferase	5.8	51.5
158	Succinate dehydrogenase	5.8	63
159	Thioredoxin	4.7	32
160	Hypothetical protein	4.84	36.6
161	Phosphoglycerate mutase	5	55
162	Oxidoreductase	5.1	46
163	pmbA protein	5	47
164	Protocatechuate-3,4-dioxygenase alpha subunit	4.74	22
165	Fumarase	5.14	56
166	Phosphoenolpyruvate carboxykinase	5.3	55
167	2-Isopropylmalate synthase	5.15	61
168	Threonyl-t-RNA synthetase	5.6	72
169	Phosphogluconate dehydratase	6	65
170	Glucose-6-phosphate dehydrogenase	6.3	55
171	Glycerin-3-phosphate dehydrogenase	6.54	36
172	Secretion protein	6.1	58
173	Acyl-CoA dehydrogenase	5.65	65.5
174	Conserved hypothetical protein Orf07902	5.74	73.7
175	Dihydroxyacid dehydratase IlvD	5.61	65.6
176	Pyrroline-5-carboxylate reductase	6.29	31.6
177	Lipodepsinonapeptide biosynthesis protein	5.55	34.9
178	Alcohol dehydrogenase, zinc containing	5.6	38.5
179	D-Hydantoinase	5.66	54.3
180	Conserved hypothetical protein	6.59	20.7
181	5-Methyltetrahydropteroyl-triglutamate-homocysteine-methyltransferase, putative	6.33	40.63

phosphate starvation-induced outer membrane protein PSI1 of *Pseudomonas fluorescens* strain AG1, synthesis of which is induced at phosphate concentrations below 0.13 mM. Inspection of the sequence of the Orf01685-specified protein predicted a hydrophobic domain similar to those of outer membrane porins, such as OprF (Brinkman *et al*., 2000). Consistent with this notion was the finding that the Orf01685-specified protein was associated with the membrane-enriched fraction obtained by sequential extraction of a lysed cell preparation (not shown). Inspection of the genomic context of Orf01685 revealed that the downstream open reading frame (ORF), Orf01683, specifies 3-oxoacyl-synthase III, which is involved in fatty acid synthesis and is activated by phosphate in *Escherichia coli* (Prescott et Vagelos, 1972), whereas the upstream ORFs, Orf01688 and Orf01690, specify a conserved hypothetical (presumably cytoplasmic) protein and an ABC efflux transporter of unknown specificity respectively. Taken together, these observations suggest that the phosphate limitation-induced protein specified by Orf01685 is most probably located in the outer membrane and may be involved in the recruitment of phosphate from the environment (Leopold *et al*., 1997), possibly in conjunction with the conserved hypothetical protein specified by the neighbouring ORF, Orf01688, which, because of its hydrophilic character, is probably a soluble protein. Genetic and biochemical experiments are needed to confirm these suggestions.

Distinct genomic expression responses of P. putida *strain KT2440 and* P. aeruginosa *strain PAO1 to iron deprivation*

Protein extracts of KT2440 and PAO1 cells grown in the presence or absence of iron were subjected to two-

dimensional electrophoresis (Figs 2 and 3 respectively), and the gels were compared. In both organisms, iron deprivation provoked the up- or downregulation of about 25 proteins; those proteins that could be identified by MALDI-TOF are summarized in Table 2. As can be seen, proteins belonging to several different physiological classes were influenced by iron starvation, but multiple proteins of only one class, that of transport and binding proteins, were upregulated in both bacteria. These included the ferripyoverdine receptor A (FpvA), the outer membrane haem receptor and the outer membrane ferrisiderophore receptor, which are regulated by the iron starvation sigma factor, PvdS, the gene of which is part of the ferric uptake regulator (Fur) protein regulon. Upregulation of fumarate hydratase, FumC, also a Fur regulon-encoded protein (Münch *et al*., 2003), was also observed in both organisms. In PAO1, 17 identified proteins, including two TonB-dependent receptors and the Fe(III) dicitrate transport protein (FecA), were upregulated, whereas three, including outer membrane protein O, a polyphosphate transporter induced by low phosphate concentrations, were downregulated in iron-starved cells. In KT2440, nine identified proteins were upregulated, whereas six, most of which, like catalase/peroxidase, dihydropyrimidine dehydrogenase and superoxide dismutase B (SodB), require iron as a cofactor, were downregulated by iron deprivation. The latter was a prominent spot in two-dimensional gels of extracts of KT2440 cells grown with iron supplementation, which was substantially reduced by growth under iron limitation (Fig. 2).

Different superoxide dismutase expression patterns in KT2440 and PAO1 in response to iron deprivation

Kim *et al*. (1999) have shown that the genes for fumarate hydratase (*fumC*) and manganese-dependent superoxide dismutase A (*sodA*) form part of a polycistronic operon, are co-transcribed and are upregulated in *P. putida* strain

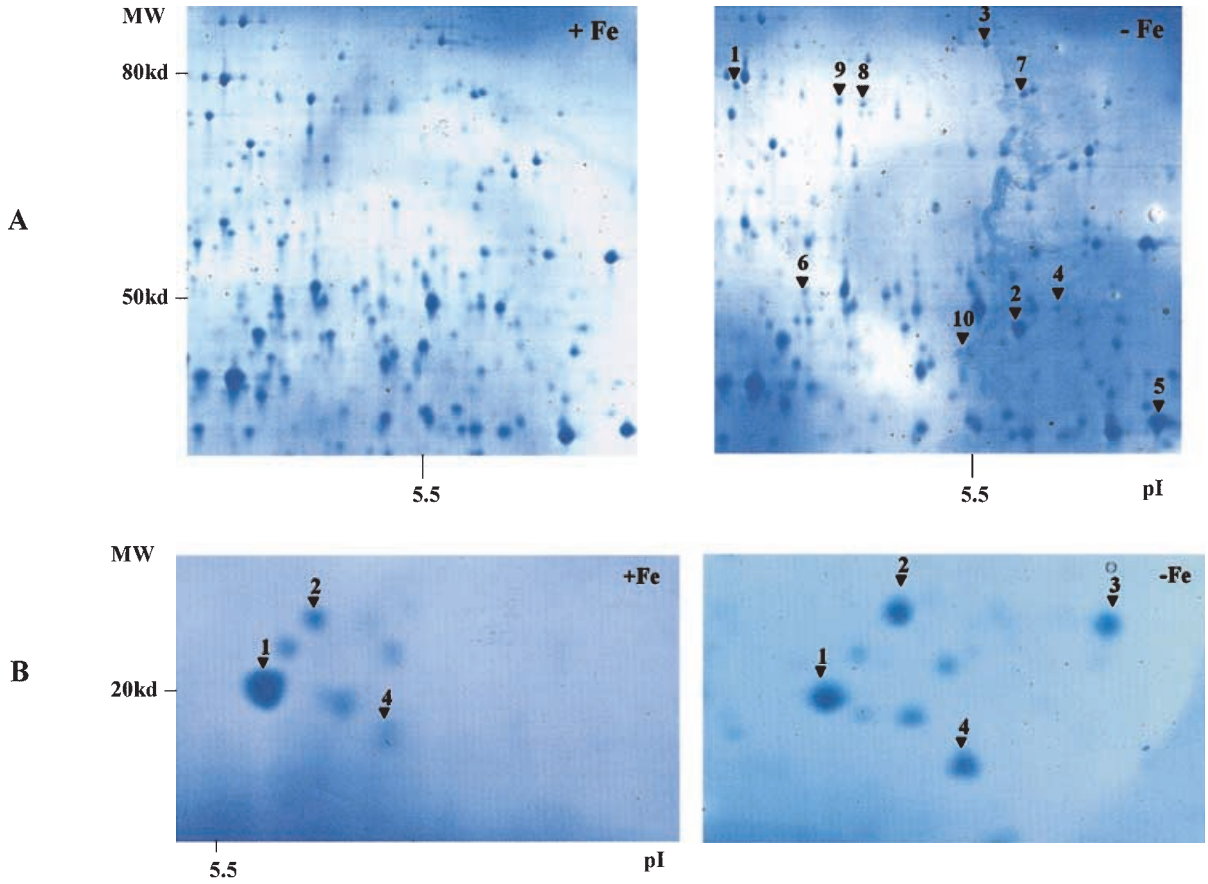

Fig. 2. Proteome response of KT2440 to iron deprivation. Relevant-high molecular-weight (A) or low-molecular-weight (B) regions of two-dimensional gels of protein extracts of KT2440 cells grown in the presence (left) or absence (right) of iron.
A. Relevant spots: 1, ferripyoverdine receptor FpvA; 2, fumarate hydratase FumC; 3, outer membrane haem receptor; 4, aspartate ammonium lyase; 5, ornithine carbamoyltransferase ArgF; 6, dihydroorotate dehydrogenase; 7, catalase/peroxidase; 8, outer membrane ferrisiderophore receptor; 9, dehydrogenase (Orf05890); 10, carbamoyl-β-alanine amidohydrolase
B. Relevant spots: 1, superoxide dismutase B (SodB); 2, hydrolase (Orf02747); 3, glutathione-*S*-transferase; 4, conserved hypothetical protein (Orf03678)

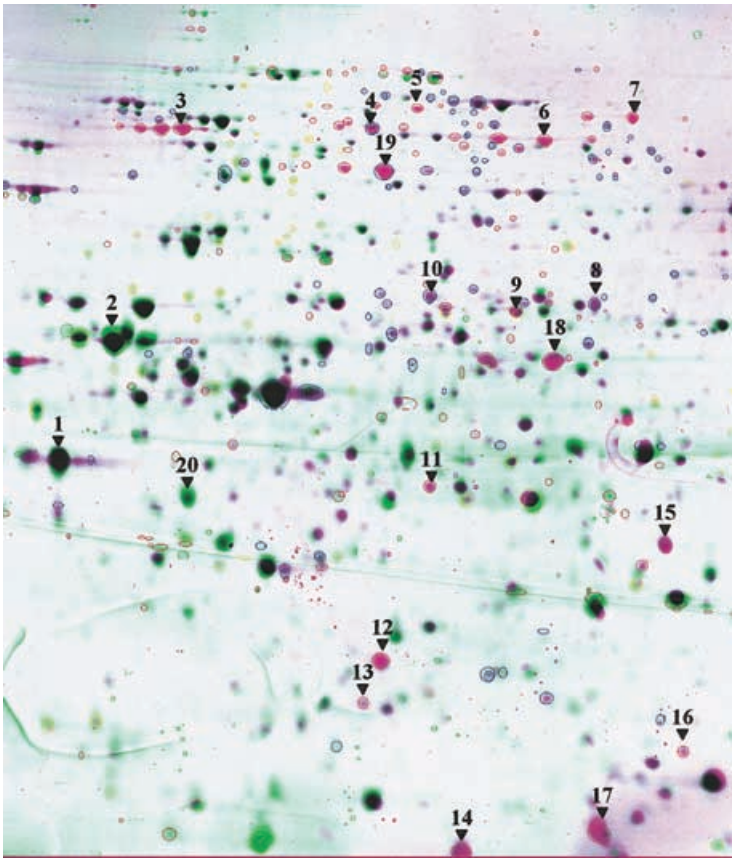

Fig. 3. Response of *Pseudomonas aeruginosa* strain PAO1 cells to iron deprivation. An image overlay, produced by the two-dimensional image analysis software Z3 (Compugen), is shown of two-dimensional gels of extracts of PAO1 cells grown in the presence (green) or absence (red) of iron. Where the spots match exactly, they are black. Spots that are upregulated in response to iron deprivation are circled in blue, those that are downregulated are circled in yellow, and those expressed only under iron deprivation are circled in red. Relevant spots: 1, outer membrane protein F; 2, outer membrane protein O; 3, ferripyoverdine receptor FpvA; 4, Fe(III) dicitrate transport protein FecA; 5, probable TonB-dependent receptor (PA4168); 6, outer membrane haemin receptor; 7, probable TonB-dependent receptor (PA0151); 8, SahH (S-adenosyl-L-homocysteine hydrolase); 9, succinate semialdehyde dehydrogenase GabD; 10, probable aldehyde dehydrogenase (PA5312); 11, probable haemin-degrading factor (PA4709); 12, hypothetical protein (PA2395); 13, probable antioxidant protein (PA3450); 14, conserved hypothetical protein (PA0423); 15, hypothetical protein (PA0660); 16, probable two-component response regulator (PA2479); 17, superoxide dismutase A; 18, fumarate hydratase FumC1; 19, Fe(III) pyocheline receptor; 20, acetoin catabolism protein AcoB.

Corvallis during iron deprivation. However, in strain KT2440, although expression of FumC was upregulated by iron deprivation, that of SodA was not detected. We therefore investigated whether these two genes are co-expressed in KT2440. Messenger RNA was isolated from cultures of KT2440 grown in medium replete with and depleted of iron, and *fumC* and *sodA* mRNAs were reverse transcribed using specific primers and amplified by polymerase chain reaction (PCR). In iron-replete medium, both genes were expressed at low levels, whereas under conditions of iron limitation, they were both strongly upregulated (Fig. 4). Curiously, although the *sodA* and *fumC* genes are upregulated by iron deprivation, and there is a correspondingly substantial increase in the FumC protein, there is no detectable increase in SodA protein and even a significant decrease in the SodB protein.

We therefore analysed Sod enzymatic activity in such cells. Protein extracts of cells of KT2440, PAO1 and the *P. putida* type strain 291T, grown in the presence or absence of iron, were fractionated on a non-denaturing polyacrylamide gel, which was subsequently stained for superoxide dismutase activity (Fig. 5). The *P. aeruginosa* PAO1 cell extracts contained two distinct Sod isoenzymes with relative activities that varied according to the iron status of the medium: in the presence of iron, only activity of the iron-dependent isoform SodB was detected, whereas in the absence of iron, the manganese-dependent isoform SodA was more active. In contrast, cell extracts of *P. putida* strains KT2440 and 291T only exhibited one type of superoxide dismutase activity, irrespective of the iron and manganese status of the growth medium. The electrophoretic mobility of this enzyme is clearly distinct from those of SodA and SodB of PAO1. Katsuwon and Anderson (1989) detected a second Sod isoenzyme in *P. putida* strain Corvallis cells cultivated on tricarboxylic acid cycle intermediates. Growth of KT2440 on succinate or citrate did not, however, induce a second isoenzyme (Fig. 5).

Superoxide dismutase in KT2440 is a SodA–SodB heterodimer

The molecular weights of the SodA and SodB protein monomers of PAO1 differ by only about 100 Da from those predicted from the KT2440 genome sequence and show high sequence similarity (96% for SodB and 86% for SodA respectively) and the same isoelectric points (pI 5.9 for SodA and pI 5.6 for SodB). Thus, it was to be expected that the KT2440 and PAO1 enzymes would have similar electrophoretic mobilities in non-denaturing gels. How-

Table 2. Comparison of proteome responses of *P. putida* KT2440 and *P. aeruginosa* PAO1 to iron limitation stress.

P.putida KT2440 protein name	expression*	*P.aeruginosa* PAO1 protein name	expression*
ferripyoverdine receptor FpvA	+	ferripyoverdine receptor FpvA	+
outer membrane ferri siderophore receptor	+	pyochelin receptor	+
outer membrane haem receptor	+	outer membrane haemin receptor	+
		tonB dependent receptor PA4168	+
		tonB dependent receptor PA0151	+
		Fe (III) di-citrate transport protein FecA	+
fumarate hydratase FumC1	+	fumarate hydratase FumC1	+
		probable aldehyde dehydrogenase PA5312	+
outer membrane protein R ompR	+	probable two-component response regulator	+
conserved hypothetical protein Orf03678	+	conserved hypothetical PA0423	+
hydrolase Orf02747	+	succinate semialdehyde dehydrogenase GabD	+
ornithine carbamoyl transferase	+		
sensory box histidine kinase NtrB	+		
		hypothetical protein PA2395	+
		hypothetical protein PA0660	+
		probable antioxidant protein PA3450	+
		NADP+ reductase Fpr	+
		nitroreductase	+
		opr O	-
		acetoin catabolism protein AcoB	-
Catalase/peroxidase	-		
Superoxide dismutase B	-		
Dihydropyrimidine dehydrogenase (Orf02168)	-	probable purine/pyrimidine phosphoribosyl transferase PA4645	-
Carbamoyl-ß-alanine amidohydrolase (Orf02175)	-		
Dehydrogenase (Orf05890)	-		
ATP dependent clp protease	-		

* change in expression: + = up regulation; - = down regulation

Colour key of protein physiological classes

signal transduction	amino acid biosynthesis
detoxification	transport and binding
Purines, pyrimidines, nucleosides, and nucleotides	energy metabolism
protein fate	regulatory functions
unknown function	conserved hypothetical protein
Biosynthesis of cofactors, prosthetic groups, and carriers	hypothetical protein
	central intermediary metabolism

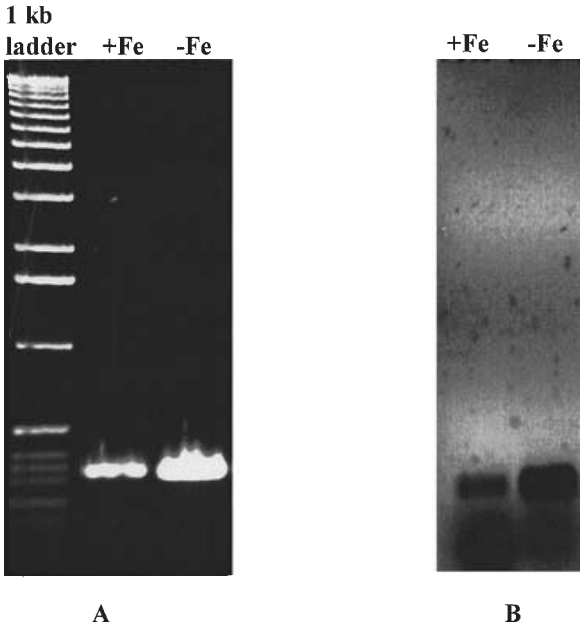

Fig. 4. Analysis of *fumC* and *sodA* locus transcription in response to iron deprivation. mRNA was isolated, reverse transcribed and PCR amplified, as described in *Experimental procedures*, and the amplicons were subjected to electrophoresis on agarose gels. *fumC* cDNA (A) and *sodA* cDNA (B) from cells grown in the presence and absence of iron.

ever, the mobility of the single active form of superoxide dismutase of KT2440 is intermediate between that of SodA and SodB of PAO1. This suggested that the superoxide dismutase of KT2440 may not be a homodimer of SodA or SodB, as generally seems to be the case (Beyer *et al.*, 1989), but rather a heterodimer of SodA and SodB monomers.

To examine this possibility, we purified Sod from KT2440 cells to homogeneity (confirmed by SDS-PAGE; not shown) and determined its N-terminal and internal peptide sequences. One portion of the purified protein was subjected to SDS-PAGE, electroblotted to a polyvinylidene difluoride (PVDF) membrane, and the N-terminal sequences determined by Edman degradation (Fig. 6). Another portion was subjected to non-denaturing PAGE, stained for Sod activity, and the active band was excised and internal peptide sequences were determined by tryptic digestion and Q-TOF mass spectrometry of the fragments obtained (Fig. 6). The resulting sequences revealed the presence of peptides from both isoenzymes in the KT2440 Sod preparation, and the quantities of amino acids released in the Edman reactions indicated a stoichiometry of 1:1 of the two isozymes. This demonstrates that Sod activity in KT2440 results from a single species consisting of a heterodimer of SodA and SodB monomers.

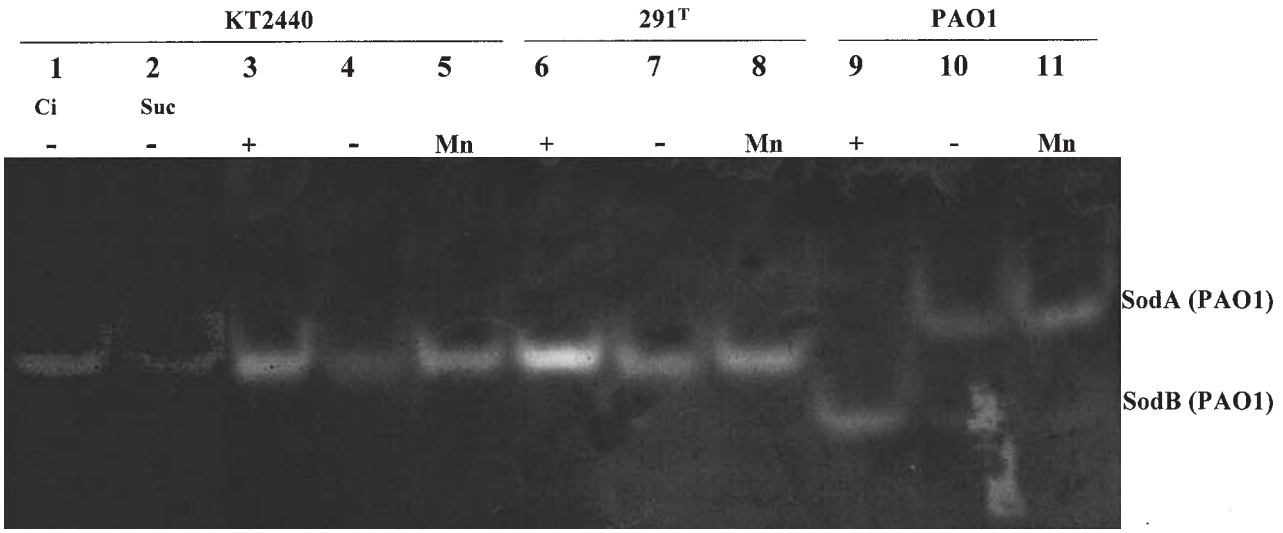

Fig. 5. Superoxide dismutase isozyme activities in *Pseudomonas* cells deprived of iron or grown with citric acid cycle intermediates. Extracts of cells of KT2440 (lanes 1–5), *P. putida* type strain 291T (6–8) and PAO1 (9–11) grown in mineral salts–glucose medium with iron supplementation (lanes 3, 6 and 9), under iron deprivation (1, 2, 4, 7 and 10), supplemented with manganese instead of iron (lane 5, 8 and 11) or grown with either citrate (1) or succinate (2) instead of glucose were fractionated on a non-denaturing 8% polyacrylamide gel, which was subsequently stained for superoxide dismutase, as described by Beauchamp and Fridovich (1971).

Discussion

In this report, we present a proteomic map of *P. putida* strain KT2440 and use it to compare its genomic response to iron starvation stress with that of *P. aeruginosa* strain PAO1. Iron is a cofactor of many enzymes, particularly respiratory enzymes and oxygenases of catabolic pathways for organic substrates (Dinkla *et al.*, 2001) and, thus, is an essential nutrient for all organisms. Iron availability varies widely in different habitats and is often a growth-limiting factor. Bacteria have evolved varied and sophisticated mechanisms to capture iron from their surroundings. It is therefore to be expected that iron deprivation will, on the one hand, provoke diverse genomic responses in different bacteria that reflect their distinct habitats and lifestyles and, on the other hand, influence their activities in different ways. *P. putida and P. aeruginosa* are both typical soil saprophytes, but differ in that *P. putida* is also a good colonizer of plant roots (see Espinosa-Urgel *et al.*, 2000), and *P. aeruginosa* is a facultative human pathogen. They therefore have distinct but overlapping lifestyles. The proteomic studies reported here revealed significant differences in the genomic responses to iron deprivation of these two organisms, which may be a reflection of their potential to lead entirely different lifestyles. Moreover, Martinez-Bueno *et al.* (2002) identified in the KT2440 genome 13 determinants of FecI extracytoplasmic function (ECF) sigma factors associated with the expression of iron acquisition systems that presumably facilitate survival under low iron conditions, whereas they found fewer such ECF determinants in the PAO1 genome. Furthermore, Cornelis and Matthijs (2002) reported differences in the TonB-dependent receptors of these two organisms that may reflect the adaptation of PAO1 to colonize mammalian tissue.

Another major difference between KT2440 and PAO1 subjected to iron deprivation concerns superoxide dismutase. PAO1 produces two distinct Sod isoenzymes, the relative activities of which varied according to the iron status of the cell: the iron-containing SodB isoform, but not the manganese-containing SodA isoform, is upregulated in cells growing in iron-replete medium, whereas the SodA isoform, but not the SodB isoform, is upregulated in cells growing in iron-depleted medium. In contrast, cell extracts of *P. putida* strains KT2440 and 291T produced only one type of superoxide dismutase, with an electrophoretic mobility on non-denaturing gels intermediate between those of SodA and SodB of PAO1, independently of the iron and manganese status of the cell. We did not detect SodA protein in two-dimensional gels of KT2440 extracts of cells grown under iron deprivation or repletion, despite observing the upregulation of expression of its mRNA. Purification of Sod protein from KT2440 cell extracts and the sequencing of its N-terminal and internal tryptic fragments revealed it to contain SodA and SodB monomers in a 1:1 ratio. Sod protein in KT2440 is thus a novel SodA–SodB heterodimer.

Sod is a crucial enzyme for bacteria that colonize eukaryotic surfaces, such as epithelial cells and plant roots, which produce activated oxygen species, such as superoxide anions, in response to bacterial infection. Katsuwon and Anderson (1989) found that the Sod enzymes

Part A: N-terminal and internal fragment sequences

```
SodA  MPTEFTDMPHTLPALPYAYDALEPHIDAQTMEIHHTKHHQTYVNGLNAAL 50
SodB  -------MAFELPPLPYAHDALQPHISKETLEYHHDKHHNTYVVNLNNLV 43

SodA  EGTEWAEWPVEKLVAAVKQLPENLRGAVTNHGGGHANHSLFWTVMSPQGG 100
SodB  PGTEFEGKTLEEIVKSS-------SGGIFNNAAQVWNHTFYWNCLSPNGG 86

SodA  GEPVGQLAQAVTAQLGGFEAFKEAFTKAALTRFGSGWAWLSVTPQKTLMV 150
SodB  GQPTGALADAINAAFGSFDKFKEEFTKTSVGTFGSGWGWLVKKADGSLAL 136

SodA  ESSGNQDSPLMHGNAPILGLDVWEHAYYLKYQNRRPEYIGAFYNVIDWVE 200
SodB  ASTIGAGCPLTSGDTPLLTCDVWEHAYYIDYRNLRPKYVEAFWNLVNWAF 186

SodA  VERRYL-EALK- 210
SodB  VAEQFEGKTFKA 198
```

Part B: Quantitation of the N-terminal amino acids released by Edman degradation

1	2	3	4	5	6	7	8	9	10
M	**P**	**T**	**E**	**F**	**T**	**D**	**M**	**?**	**H**
6.2	5.0	3.7	3.9	2.7	2.4	2.0	2.2		1.2
M	***A***	***F***	***E***	***L***	***P***	***P***	***L***	***P***	***Y***
5.7	4.9	3.1	4.0	2.9	2.4	2.1	1.4	1.6	1.6

11	12	13	14	15	16	17	18	19	20
T	**L**	**P**	**A**	**L**	**P**	**Y**	**A**	**Y**	**D**
0.8	0.2	1.3	0.2	0.6	0.9	0.4	0.5	1.0	1.3
A	***H***	***D***	***?***	***L***	***Q***	***P***	***H***	***I***	***S***
0.8	0.4	0.7		0.7	0.6	0.5	0.5	1.0	1.0

21	22	23	24	25
A	**L**	**E**	**P**	**H**
0.8	0.3	0.6	0.4	0.1
K	***E***	***T***	***?***	***E***
0.8	0.5	0.6		0.1

Fig. 6. SodA and SodB sequences found in superoxide dismutase purified from KT2440.
A. The Sod polypeptide sequences are aligned. Identical amino acids are indicated by shading. The N-terminal sequences of the purified active Sod protein are shown in red; those of the internal fragments are shown in blue.
B. The amino acids are numbered left to right from the N-terminus (upper line). The upper sequence is that of SodA, the lower sequence in italics is that of SodB. The amounts in pmol of amino acids recovered after Edman degradation are given in the lower line. As can be seen, there was essentially stoichiometric recoveries of equivalent amino acids from the SodA and SodB polypeptides.

of a plant pathogenic strain of *Pseudomonas syringae* and a non-pathogenic strain of *P. putida* had different electrophoretic mobilities. It is possible that the various differences in superoxide dismutases observed in this report and others may reflect the different lifestyles of the corresponding organisms, whether and how they interact with eukaryotic organisms, and the nature, types and quantities of active oxygen species with which they are faced. Genetic mix and match experiments may provide further insights into the functional basis of this diversity.

Experimental procedures

Bacterial strains and culture conditions

Pseudomonas putida strain KT2440 (Bagdasarian *et al.*, 1981; Regenhardt *et al.*, 2002), *P. putida* type strain 291T (DSMZ) and *P. aeruginosa* strain PAO1 (Stover *et al.*, 2000) cells were cultivated at 30°C in low-phosphate mineral salts medium (50 mM MOPS, 50 mM KCl, 0.8 mM $MgSO_4$, 0.8 mM $CaCl_2$, 0.3 mM KH_2PO_4, 0.5 g l^{-1} sodium citrate, 1 g l^{-1} $(NH_4)_2SO_4$, 36 μM $FeSO_4$) supplemented with 10 mM glucose, 20 mM citrate or 20 mM succinate as carbon source. For iron limitation experiments, overnight cultures were transferred to fresh medium to a final turbidity at 600 nm of 0.05, with or without iron supplementation, and grown overnight (turbidity 1.5). Cells were harvested by centrifugation (7000 *g*, SS34 rotor, Sorvall centrifuge) and washed twice with phosphate-buffered saline (PBS, pH 7.4).

Two-dimensional gel electrophoresis

Sample preparation. Cell material (10 mg wet weight) was suspended in 1 ml of reswelling solution for isoelectric focusing (IEF), consisting of 7.4 M urea, 2 M thiourea, 4% CHAPS, 30 mM dithiothreitol (DTT), 20 mM Trizma base, protease inhibitor cocktail [one tablet of Mini(tm) Complete per 20 ml solution; Roche], and the suspension was sonicated for 6 × 30 s on ice (1 s repeating duty cycle, Labsonic U; Braun). Protein concentrations were determined by the procedure of Bradford (1976).

Electrophoresis. Two-dimensional gel electrophoresis was carried out according to the method of Görg (1991). About

300 μg of protein was applied by reswelling to dry pH 4–7 IPG ReadyStrips (Bio-Rad). IEF was performed in a Protean IEF cell from Bio-Rad at the maximum voltage of 5000 V for 150 kVh in total. The strips were then transferred to 1.5-mm-thick 12–15% gradient SDS-polyacrylamide gels and developed overnight in the IsoDalt system from Amersham Pharmacia Biotech. The gels were stained using colloidal Coomassie R-250 or silver stain, according to the method of Blum *et al*. (1987).

Mass spectrometric analysis

Protein spots of interest were excised, destained and the samples prepared for MALDI-TOF analysis according to the method of Wissing *et al*. (2000). The peptide fingerprints obtained were used in database searches in our in-house KT2440 protein database based on the genome sequence. Internal peptide sequences were determined by Q-TOF analysis. The samples were prepared according to the methods of Wissing *et al*. (2000).

Image analysis

Differential protein expression was visualized with the Z3 image analysis software (Compugen; http://www.2dgels.com).

Superoxide dismutase activity test

Strains were cultivated overnight in standard mineral salts medium, with 10 mM glucose, 20 mM citrate or 20 mM succinate as carbon source, respectively, with or without the addition of 36 μM $FeCl_2$ or $MnCl_2$, and the cells were harvested, washed with PBS buffer and resuspended in 50 mM Tris buffer, pH 7.4. A suspension representing 1.5 ml of original culture was disrupted by sonication, cell debris was removed, and 20 μl of the crude enzyme extracts were loaded on an 8% non-denaturing polyacrylamide gel and subjected to electrophoresis. Gels were subsequently stained for superoxide dismutase as described by Beauchamp and Fridovich (1971).

RT-PCR of sodA *and* fumC *transcripts*

Pseudomonas putida strain KT2440 cells, grown in medium containing or lacking added iron, were harvested in the exponential growth phase by centrifugation (17 000 *g* for 2 min at 4°C). The pellet was resuspended in 0.25 ml of ice-cold 0.9% NaCl and mechanically disrupted in a bead beater containing 0.3 g of glass beads (0.17–0.18 mm; Braun Biotech) at 4000 r.p.m. for 30 s. Cell lysis was completed by the addition of 0.75 ml of TRI Reagent LS (Sigma), and RNA was extracted according to the manufacturer's instructions. An aliquot of the RNA (4.2 μg or 3.3 μg from cells grown with or without iron respectively), 2 pmol of primers R-sodA (5′-GGT TGCCGCTACTCTCCACCATCA-3′) and R-fumC (5′-AGC GAAACCGACAGCGAACTGC-3′), and 10 nmol of dNTP in 12 μl were heated to 65°C for 5 min then chilled on ice. First-strand synthesis was carried out at 42°C for 50 min, after the addition of 200 U of Superscript II (Invitrogen), in 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT and 20 U of SuperaseIn RNase inhibitor (Ambion) in 20 μl reactions. Reaction tubes were then heated to 70°C for 15 min, and 2 μl of the reaction mixture was used in 50 μl PCR reactions for amplification of a 430 bp product of *sodA* (positions 37–466) and a 320 bp product of *fumC* (positions 407–726), with primer pairs F-sodA (5′-CCTGCATTGCCATACGCCTACGA-3′) and R-sodA, or F-fumC (5′-ATGACATCATTCCGACCAC CATCC-3′) and R-fumC respectively. PCRs contained 2 U of *Taq* DNA polymerase, buffer and Q-solution provided by Qiagen, 10 μM both primers and 0.2 mM each dNTP. Thermal conditions were 5 min at 94°C, 30 cycles of 30 s at 94°C, 30 s at 61°C and 1 min at 72°C, and 7 min at 72°C. The amplicons were analysed by electrophoresis on 1% agarose gels in TAE buffer.

Purification of superoxide dismutase from KT2440

Cells of *P. putida* strain KT2440 were cultured at 30°C overnight (late log phase) with gentle agitation in 2 l flasks containing 500 ml of medium supplemented or not with Fe, as described above, harvested by centrifugation for 30 min at 4500 *g*, frozen and stored at −20°C. Frozen cells were thawed, suspended in a twofold volume of buffer A (50 mM Tris-HCl, pH 7.0) containing one protease inhibitor cocktail tablet (Roche) and 0.4 mg ml^{-1} DNase I, grade II (Boehringer Mannheim), and disrupted by sonication (4 × 60 s). The cell extract was centrifuged at 35 000 *g* for 35 min at 4°C, and the supernatant fluid was stored at 4°C. Superoxide dismutase from supernatant fluid corresponding to 1 l of original culture was purified by preparative non-denaturing PAGE (5–15% polyacrylamide gradient), at 45 V constant power at 4°C, according to the manufacturer's (Bio-Rad) protocol. The gel region containing active Sod protein, detected in a parallel track by activity staining (Beauchamp and Fridovich, 1971), was excised, suspended in two volumes of buffer A and homogenized in a glass tissue homogenizer. The eluate, obtained after removal of polyacrylamide by centrifugation at 4500 *g* at 4°C for 15 min, was concentrated by ultrafiltration on a Centricon YM10 system (Amicon) and applied to an ion-exchange chromatography Mono-Q HR 5/5 column equilibrated with buffer A. Proteins were eluted with an increasing gradient of 0–1 M NaCl in the same buffer. The flow rate was 0.5 ml min^{-1}, and fractions of 0.25 ml were collected. The Sod was detected by SDS-PAGE, and non-denaturing PAGE followed by activity staining, in fractions eluting in 0.2–0.35 M NaCl. Active fractions were pooled, dialysed against buffer B (50 mM Tris-HCl, pH 7.0, 0.15 M NaCl) at 4°C and concentrated by ultrafiltration to 1 ml. The concentrated sample was applied to a Superose 12 HR 10/30 gel filtration column equilibrated with buffer B and maintained at 4°C. The column was developed with buffer B at a flow rate of 1 ml min^{-1}. Fractions (0.4 ml) were collected and analysed for Sod as above. The following standards were used to calibrate the column: ribonuclease A (13.7 kDa), chymotrypsinogen A (25 kDa), ovalbumin (43 kDa), bovine serum albumin (67 kDa). The purified Sod was dialysed against buffer A at 4°C and subsequently stored at −20°C. N-terminal and internal peptide sequencing was performed to confirm the identity and purity of the protein.

Acknowledgements

M.F. is grateful to the European Community for awarding him a Marie Curie postdoctoral fellowship, and K.T. thanks the Fonds der Chemischen Industrie for their generous support. This work was funded by grant 311756 provided by the German Ministry of Education and Research.

Supplementary material

Additional information on the proteome 2-D gel spots (protein name, molecular weight and pI value where known) can be accessed by clicking on the spots in the reference gel corresponding to Fig. 1, available at http://www.blackwellpublishing.com/products/journals/suppmat/emi/emi465/emi465sm.htm

References

Bagdasarian, M., Lurz, R., Rückert, B., Franklin, F.C.H., Bagdasarian, M.M., Frey, J., and Timmis, K.N. (1981) Specific-purpose plasmid cloning vectors. II. Broad host range, high copy number, RSF1010-derived vectors, and a host-vector system for gene cloning in *Pseudomonas*. *Gene* **16:** 237–247.

Beauchamp, C., and Fridovich, I. (1971) Superoxide dismutase: improved assays and assay application to acrylamide gels. *Anal Biochem* **44:** 276–287.

Beyer, W.F., Jr, Reynolds, J.A., and Fridovich, I. (1989) Differences between the manganese- and the iron-containing superoxide dismutases of *Escherichia coli* detected through sedimentation equilibrium, hydrodynamic, and spectroscopic studies. *Biochemistry* **28:** 4403–4409.

Blum, H., Beier, H., and Gross, H.J. (1987) Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis* **8:** 93–99.

Bradford, M.M. (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72:** 248–254.

Brinkman, F.S., Bains, M., and Hancock, R.E. (2000) The amino terminus of *Pseudomonas aeruginosa* outer membrane protein OprF forms channels in lipid bilayer membranes: correlation with a three-dimensional model. *J Bacteriol* **182:** 5251–5255.

Bühler, B., Witholt, B., Hauer, B., and Schmid, A. (2002) Characterization and application of xylene monooxygenase for multistep biocatalysis. *Appl Environ Microbiol* **68:** 560–568.

Cases, I., Velazquez, F., and de Lorenzo, V. (2001) Role of ptsO in carbon-mediated inhibition of the Pu promoter belonging to the pWW0 *Pseudomonas putida* plasmid. *J Bacteriol* **183:** 5128–5133.

Cordwell, S.J., Nouwens, A.S., and Walsh, B.J. (2001) Comparative proteomics of bacterial pathogens. *Proteomics* **1:** 461–472.

Cornelis, P., and Matthijs, S. (2002) Diversity of siderophore-mediated iron uptake systems in fluorescent pseudomonads: not only pyoverdines. *Environ Microbiol* **4:** 787–798.

Dinkla, I.J., Gabor, E.M., and Janssen, D.B. (2001) Effects of iron limitation on the degradation of toluene by *Pseudomonas* strains carrying the tol (pWWO) plasmid. *Appl Environ Microbiol* **67:** 3406–3412.

Espinosa-Urgel, M., Salido, A., and Ramos, J.L. (2000) Genetic analysis of functions involved in adhesion of *Pseudomonas putida* to seeds. *J Bacteriol* **182:** 2363–2369.

Federal Register (1982) Appendix E, Certified host–vector systems. 47: 17197.

Görg, A. (1991) Two-dimensional electrophoresis. *Nature* **349:** 545–546.

Kasai, Y., Inoue, J., and Harayama, S. (2001) The TOL plasmid pWW0 xylN gene product from *Pseudomonas putida* is involved in m-xylene uptake. *J Bacteriol* **183:** 6662–6666.

Katsuwon, J., and Anderson, A.J. (1989) Response of plant-colonizing pseudomonads to hydrogen peroxide. *Appl Environ Microbiol* **55:** 2985–2989.

Kim, Y.C., Miller, C.D., and Anderson, A.J. (1999) Transcriptional regulation by iron of genes encoding iron- and manganese-superoxide dismutase from *Pseudomonas putida*. *Gene* **239:** 129–135.

Klinke, S., Dauner, M., Scott, G., Kessler, B., and Witholt, B. (2000) Inactivation of isocitrate lyase leads to increased production of medium-chain-length poly (3-hydroxyalkanoates) in *Pseudomonas putida*. *Appl Environ Microbiol* **66:** 909–913.

Leopold, K., Jacobsen, S., and Nybroe, O. (1997) A phosphate-starvation-inducible outer-membrane protein of *Pseudomonas fluorescens* Ag1 as an immunological phosphate-starvation marker. *Microbiology* **143:** 1019–1027.

Llamas, M.A., Ramos, J.L., and Rodriguez-Herva, J.J. (2003) Transcriptional organization of the *Pseudomonas putida* tol-oprL genes. *J Bacteriol* **185:** 184–195.

Martinez-Bueno, M.A., Tobe, S.R., Rey, M., and Ramos, J.L. (2002) Detection of multiple extracytoplasmic function (ECF) sigma factors in the genome of *Pseudomonas putida* KT2440 and their counterparts in *Pseudomonas aeruginosa* PA01. *Environ Microbiol* **4:** 842–855.

Münch, R., Hiller, K., Barg, H., Heldt, D., Linz, S., Wingender, E., and Jahn, D. (2003) PRODORIC: prokaryotic database of gene regulation. *Nucleic Acids Res* **31:** 266–269.

Nelson, K.E., Weinel, C., Paulsen, I.T., Dodson, R.J., Hilbert, H., Martins Dos Santos, V., *et al.* (2002) The complete genome sequence of *Pseudomonas putida* KT2440: insights into diversity and virulence of the Pseudomonads. *Environ Microbiol* **4:** 799–808.

Prescott, D.J., and Vagelos, R.P. (1972) Acyl carrier protein. *Adv Enzymol Relat Areas Mol Biol* **36:** 269–311.

Regenhardt, D., Heuer, H., Heim, S., Fernandez, D.U., Strömpl, C., Moore, E.R.B., and Timmis, K.N. (2002) Pedigree and taxonomic credentials of *Pseudomonas putida* strain KT2440. *Environ Microbiol* **4:** 912–915.

Ronchel, M.C., and Ramos, J.L. (2001) Dual system to reinforce biological containment of recombinant bacteria designed for rhizoremediation. *Appl Environ Microbiol* **67:** 2649–2656.

Stover, C.K., Pham, X.Q., Erwin, A.L., Mizoguchi, S.D., Warrener, P., Hickey, M.J., *et al.* (2000) Complete genome sequence of *Pseudomonas aeruginosa* PA01, an opportunistic pathogen. *Nature* **406:** 959–964.

Timmis, K.N. (2002) *Pseudomonas putida*: a cosmopolitan opportunist par excellence. *Environ Microbiol* **4:** 779–781.

Wissing, J., Heim, S., Flohè, L., Bilitewski, U., and Frank, R. (2000) Enrichment of hydrophobic proteins via Triton X-114 phase partitioning and hydroxyapatite column chromatography for mass spectrometry. *Electrophoresis* **21:** 2589–2593.