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Proteins Induced by Sulfate Limitation in *Escherichia coli*, *Pseudomonas putida*, or *Staphylococcus aureus*

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Two-dimensional gel electrophoresis of proteins from *Escherichia coli*, *Pseudomonas putida*, and *Staphylococcus aureus*, grown with methionine or one of a variety of organosulfates and organosulfonates as the sole source of sulfur, showed expression of specific sets of 7 to 14 proteins which were not observed during growth with sulfate or cysteine for all three species or with thiocyanate for *P. putida* and *S. aureus*. Under the same conditions, arylsulfatase activity in *P. putida* and *S. aureus* was seen to increase by up to 140-fold, suggesting that the proteins induced under these conditions may be involved in sulfur metabolism. We propose that these proteins are members of a sulfate starvation-induced stimulon.

All bacteria display an absolute growth requirement for sulfur, which is needed for synthesis of both protein and a variety of essential cofactors and which makes up about 1% of the dry weight of the cell (16). Although total sulfur in natural environments is rarely growth limiting, much sulfur in soil is present in a combined form as sulfate esters or as sulfonates and is not available as the inorganic sulfate which predominates in oceans and rivers (8). Biosynthesis of cysteine occurs primarily via assimilatory metabolism of sulfate and is regulated by the levels of cysteine, sulfide, and sulfate present (13, 14, 20). Full induction of the cysteine biosynthetic pathway occurs only under conditions of sulfur limitation, which are usually provided by supplying djenkolate or glutathione as the sole sulfur source for growth (13, 14). The cysteine regulon is completely repressed by sulfide, which interferes with the binding of the coinducers of the pathway, *O*-acetylserine and the CysB protein, to the promoters concerned (20), and by cysteine, which acts as an end product inhibitor of acetylserine synthesis (14). In contrast, sulfate reduces expression of the cysteine biosynthetic enzymes to 40 to 50% of fully derepressed levels in *Escherichia coli* (22), whereas in *Salmonella typhimurium* little or no effect is observed (14).

Sulfur limitation has also been shown to stimulate other sulfur metabolic systems. An increase in sulfate transport has been observed for several organisms (15, 19, 21), and stimulation of sulfonate uptake has also been previously reported (3). Arylsulfatase activity is increased by sulfate limitation in several bacterial species (6). In *Klebsiella aerogenes*, growth of cells with methionine as the sole sulfur source leads to a 90-fold increase in arylsulfatase activity (1), and an even greater effect is found in *Pseudomonas* sp. $C_{12}B$ (9). Broad-spectrum arylsulfatase activity is observed for *Pseudomonas aeruginosa* after growth with a variety of sulfate esters as the sole sulfur source and also when alternative sulfur sources such as taurine, cysteic acid, cysteine sulfonate, and cysteine sulfinate are used (10). In contrast, when sulfate, sulfite, thiosulfate, or cysteine is present, no enzyme is produced, suggesting that intermediates of the assimilatory pathway act to repress synthesis of sulfatase (10).

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Global regulatory systems which respond to limitation for carbon, nitrogen, or phosphorus are well known (11, 18) and control the levels of a large number of proteins involved in the metabolism of the element concerned. In *E. coli*, these proteins have been characterized by two-dimensional polyacrylamide gel electrophoresis of cell extracts obtained after growth under a number of conditions (26). The carbon, nitrogen, and phosphorus starvation responses consist of 25, 31, and 55 specific proteins, respectively; a number of other proteins are also induced as part of a general starvation response (26). By analogy to these systems, and particularly to the phosphate starvation-induced stimulon, we anticipated that sulfur metabolism might be controlled, at least in part, by a similar mechanism dependent on sulfate levels.

Pseudomonas putida S313 (DSM 8334) was isolated previously (30), *Staphylococcus aureus* 80Cr5 (7) was kindly made available by B. Berger-Bächi, University of Zürich, and *E. coli* MC4100 (5) was obtained from G. Weinstock, University of Texas. All organisms were routinely grown in a salts medium containing 50 mM Tris-HCl (pH 7.3), 25 mM carbon source (glucose or succinate), 20 mM NH_4Cl, 2 mM potassium phosphate, 0.5 mM $MgCl_2$, and 1 ml of a trace element solution per liter (24). For *S. aureus*, this growth medium was supplemented both with thiamine and nicotinic acid (1 μg/ml) and with the amino acids Pro, Arg, Val, Leu, Ile, Asp, Glu, Ala, His, Gly, Phe, Ser, Tyr, Trp, and Lys (each at 100 μg/ml). Sulfur sources (Fluka, Buchs, Switzerland; all with purity of >99%) were added as described in the text (500 μM). All glassware used in sulfate-limited experiments was washed with 3 M HCl and rinsed thoroughly with distilled water before use. Cultures were grown aerobically on a rotary shaker at 37°C (*E. coli* and *S. aureus*) or 30°C (*P. putida*). Growth was monitored spectrophotometrically at 650 nm, and cells were harvested in the mid-exponential phase. Two-dimensional gel electrophoresis of total cell extracts (5 to 20 μg of protein) was carried out according to the method described by Hochstrasser et al. (12), and the gels were silver stained (4) and dried in cellophane. Arylsulfatase was assayed in whole cells in 100 mM Tris-acetate buffer (pH 9.0) with 4-nitrocatechol-sulfate (10 mM) as the substrate and with colorimetric (515 nm; $\varepsilon = 13\ mM^{-1}$) monitoring of nitrocatechol production; the reaction (0.5 ml) was started by the addition of cell suspension (1 to 4 mg of protein).

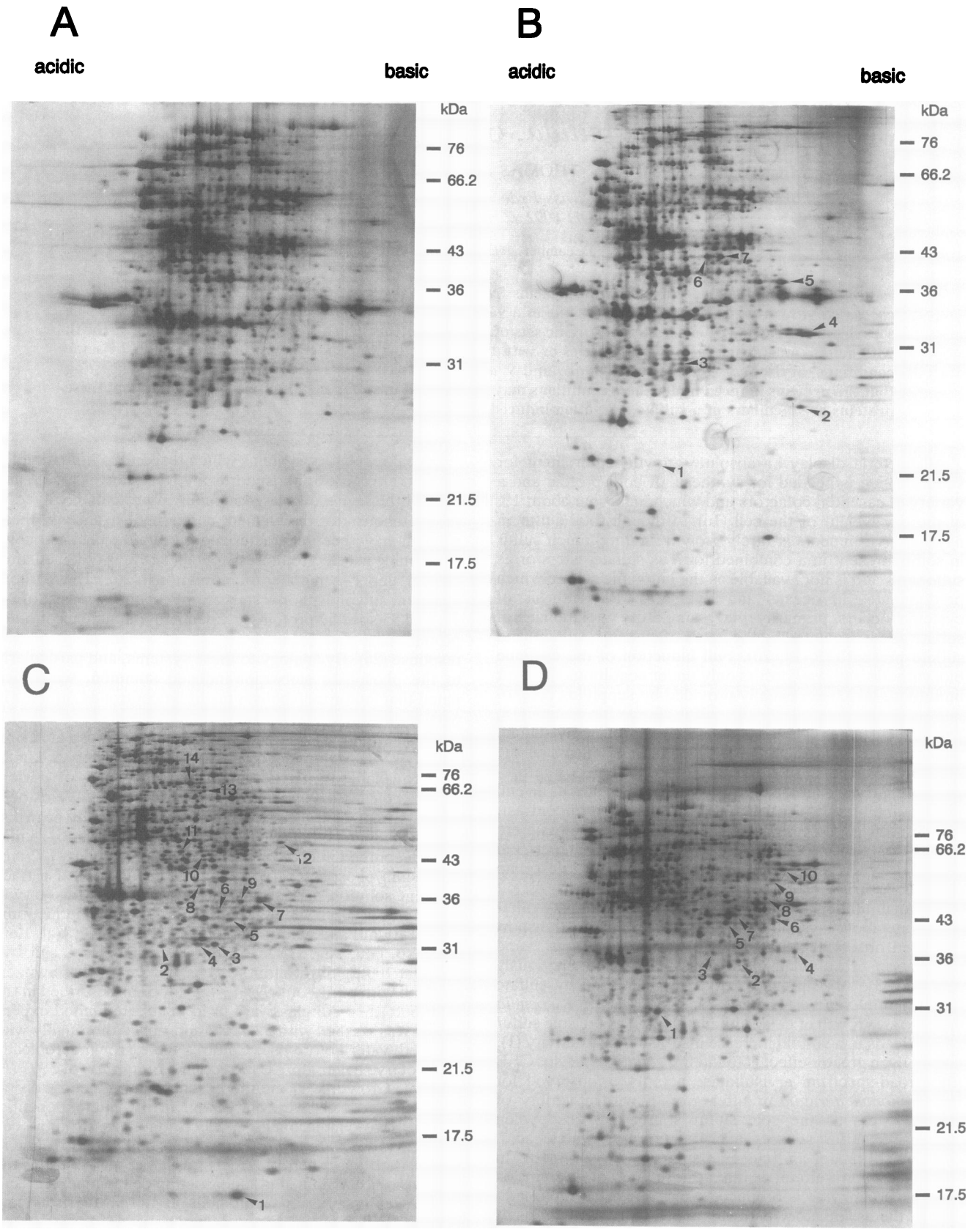

FIG. 1. Two-dimensional electropherograms of total cell protein from microorganisms grown aerobically in a minimal salts medium with different sulfur sources. (A) *E. coli* (cysteine or sulfate); (B) *E. coli* (SSI substrates); (C) *P. putida* (SSI substrates); (D) *S. aureus* (SSI substrates). The arrows indicate proteins which are strongly induced under SSI conditions. Sulfur substrates leading to the SSI response in each organism are listed in Table 2.

Changes in protein composition during growth with various sources of sulfur. We first showed that *E. coli*, *P. putida*, and *S. aureus* can grow with a variety of sulfur-containing compounds as the sole sources of sulfur, including cysteine, methionine, thiocyanate, organosulfates, alkyl organosulfonates, and, in one case, aryl organosulfonates (see Table 2). Using two-dimensional gel electrophoresis, we then investigated the protein compositions of these organisms when one of nine sulfur-containing compounds is utilized as the sole source of sulfur (Fig. 1). Visual comparison of the protein patterns obtained revealed a consistent protein pattern for *E. coli* when the sulfur source was sulfate or cysteine (Fig. 1A). When any one of the alternative sources of sulfur was used (see Table 2), the appearance of a set of seven polypeptides was observed (Fig. 1B; Table 1); these proteins were absent when sulfate or cysteine had been used for growth. This phenomenon was also observed for *P. putida* (14 additional polypeptides observed) and for *S. aureus* (10 polypeptides) (Fig. 1C and D; Table 1). For the latter two organisms, thiocyanate also functioned in a manner similar to that of sulfate. *E. coli* did not grow well either with this compound as the sulfur source or with alkyl or aryl organosulfates. When the organisms were grown with 500 μM sulfate as the sulfur source, harvested, washed, and then incubated for 1 h in a sulfate-free medium, the same sets of proteins, which we term SSI (sulfate starvation-induced) proteins, were also seen to be synthesized.

Large numbers of phosphorus, nitrogen, and carbon starvation-induced proteins from *E. coli* K-12 have already been identified elsewhere (26) and classified by the alphanumeric system described by Pederson et al. (23). The SSI proteins reported here for *E. coli* (Table 1) do not correlate with general starvation proteins already registered in the data base. They also represent a minimum number, because only proteins which were not detectable at all by silver staining after growth with sulfate or cysteine were included. Enzymes involved in cysteine biosynthesis are poorly represented in the *E. coli* data base (26); however, since they show significant levels of expression during growth with sulfate (14, 22), they are unlikely to be among the SSI proteins listed (Table 1). The regulatory protein of the cysteine regulon, CysB, has been reported to have a molecular weight of 39,000 and a neutral pI (17) and may perhaps correspond to the H38.0 SSI protein of *E. coli* (Table 1), although the intensity of the protein spot argues against this (the CysB protein of *S. typhimurium* has been identified by two-dimensional gel electrophoresis and is present at only low levels relative to other cellular proteins [2]). The expression of a number of other polypeptides appeared by visual comparison to be enhanced under SSI conditions, but reliable quantification was not possible with the silver-stained protein gels alone.

Regulation of arylsulfatase during growth on various sulfur sources. The induction of enzymes involved in phosphorus metabolism under conditions of phosphorus starvation has been previously well characterized (25). By analogy, it was expected that proteins whose expression is enhanced by sulfate limitation may represent enzymes involved in scavenging sulfur. Arylsulfatase has previously been shown to be regulated by a derepressive mechanism in *K. aerogenes* (1) and in *Pseudomonas* species (9, 10); the absence of sulfate or cysteine leads to enhanced enzyme synthesis (1, 9). Although *E. coli* possesses a latent arylsulfatase, it is expressed only when the adjacent tyramine oxidase gene is induced and is not directly controlled by the sulfur supply (29). We therefore measured 4-nitrocatecholsulfate cleavage in whole cells of *P. putida* and *S. aureus* after growth with various sole sources of sulfur. Sulfatase activity was low when sulfate, cysteine, or thiocyanate (or glutathione [for *S. aureus*]) was the sole source of sulfur during growth, but the enzyme was present at much higher levels after growth with other sulfur sources (Table 2), corresponding exactly with the SSI effect observed on the two-dimensional gels. Growth with cysteine or sulfate in addition to nitrocatecholsulfate also led to low levels of the enzyme (Table 2); addition of sulfate, cysteine, or thiocyanate (500 μM) to the assay did not inhibit the enzyme (data not shown). Enzyme levels were very dependent on the growth phase for both organisms.

TABLE 1. Locations on two-dimensional protein gels of proteins induced under SSI conditions for *P. putida*, *E. coli*, and *S. aureus*[a]

Reference no.[b]	*P. putida*	*E. coli*	*S. aureus*
1	G11.0	B21.9	D29.7
2	D30.0	H25.7	F37.1
3	G30.4	C29.3	E37.9
4	F31.0	H32.3	G38.5
5	F33.2	H38.0	E43.2
6	F34.3	F42.5	G44.1
7	G35.2	F42.5	F44.9
8	F38.1		G47.7
9	G35.9		G51.4
10	F42.8		G54.0
11	E43.6		
12	H44.6		
13	F54.6		
14	F56.5		

[a] Alphanumeric values according to the system described by Pederson et al. (23). Each letter (A to G) gives an approximate value for pI; each number represents the molecular mass of the protein concerned (in kilodaltons), as read from the two-dimensional gel.
[b] The reference numbers correspond to the numbers given in Fig. 1.

TABLE 2. Arylsulfatase activity and induction of the SSI stimulon after growth of *P. putida*, *E. coli*, and *S. aureus* with different sulfur sources

Sulfur source for growth (500 μM)	Relative arylsulfatase activity[a,b]		
	P. putida	*E. coli*	*S. aureus*
Sulfate	0.2 (−)	(−)	2.2 (−)
Cysteine	1.0 (−)	(−)	1.0 (−)
Methionine	38 (+)	(+)	116 (+)
Thiocyanate	0.1 (−)	NG[c]	1.9 (−)
Glutathione	NG	(+)	2.5 (−)
Dodecyl sulfate	124 (+)	NG	136 (+)
4-NCS[d]	64 (+)	NG	137 (+)
Ethanesulfonate	101 (+)	(+)	95 (+)
Benzenesulfonate	141 (+)	NG	NG
4-NCS + sulfate	0.5	NA[e]	NA
4-NCS + cysteine	3.0	NA	NA
4-NCS + thiocyanate	0.4	NA	NA

[a] Enzyme activity normalized such that activity after growth with cysteine equals 1.0. No arylsulfatase activity was observed for *E. coli* (see reference 29). Enzyme activities obtained after growth on 4-nitrocatecholsulfate were 8 to 20 mkat kg^{-1}.
[b] Presence (+) or absence (−) of the SSI proteins in two-dimensional protein electropherograms of total cell protein is indicated in parentheses.
[c] NG, poor growth or no growth observed.
[d] 4-NCS, 4-nitrocatecholsulfate.
[e] NA, not assayed.

Derepression of a set of SSI proteins is observed in both gram-positive and gram-negative microorganisms and may therefore represent a general response to sulfate limitation. Arylsulfatase is clearly regulated as part of this stimulon, but the nature of the other SSI proteins has yet to be explored. In the phosphate starvation-induced stimulon, conditions of phosphate limitation lead to derepression of high-affinity transport systems for phosphate and sugar phosphates (25) and of degradative enzymes such as alkaline phosphatase (28) and carbon-phosphorus lyase (27). We therefore expect the SSI proteins to include enzymes that are important for the scavenging of sulfur-containing compounds, possibly including sulfatases, sulfonatases, enzymes required for degradation of methionine and taurine, and corresponding transport proteins, and we are currently investigating their identities and regulation in more detail.

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