Effect of Iron Limitation on Growth, Siderophore Production, and Expression of Outer Membrane Proteins of *Vibrio cholerae*

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Vibrio cholerae strains secrete a phenolate-type siderophore when grown in low-iron medium. The siderophore was detected as early as 3.5 h after downshift to iron-poor medium, and it continued to accumulate in the medium as the cells entered stationary phase. Two clinical isolates and an environmental isolate were examined for the amount of siderophore produced. The environmental isolate produced more siderophore and continued to secrete it at concentrations of iron that repressed synthesis in the clinical isolates. Concomitant with production of siderophore, at least six new proteins were seen in the outer membranes of iron starved cells. One of the proteins was large (220,000 M_r [220K]) and appeared to be loosely associated with the outer membrane. The other five proteins had approximate M_r values of 77K, 76K, 75K, 73K, and 62K. The 62K protein, like the 40K major outer membrane protein, was heat modifiable. One or more of these proteins may be a component of the receptor for the iron-siderophore complex.

Cholera may result from infection with either classical or El Tor biotypes of *Vibrio cholerae*. During outbreaks of the disease, the vibrios can be isolated from patients and from sewage-contaminated water (9). Recent studies have shown that *V. cholerae* El Tor strains can also be found in coastal waters in areas free of known cholera cases (15). Both of these environments, marine areas and animal hosts, have limited amounts of free iron available for microbial growth since the metal forms insoluble complexes at neutral or alkaline pH and is sequestered in hosts by iron-binding proteins (18). Most of the iron in mammalian hosts is found in hemoglobin or ferritin. The small quantity of extracellular iron in serum and secretions is held by the high-affinity iron-binding proteins transferrin and lactoferrin (33). Like many other microorganisms, *V. cholerae* produces an iron chelator, or siderophore (18), which serves to solubilize and transport iron into the cell under conditions of iron limitation. *V. cholerae* produces a phenolate-type siderophore (25) that is similar to, but distinct from, enterochelin (enterobactin), the siderophore synthesized by *Escherichia coli* K-12 (5), *Salmonella typhimurium* (27, 34), and *Shigella sonnei* (26).

When iron is limiting, *E. coli* K-12 not only produces the siderophore but also synthesizes at least three outer membrane proteins which are less abundant in cells grown with sufficient iron (4, 23). One of these proteins, 81K (4), is the

receptor for ferrienterochelin (12, 21, 28). The appearance of these proteins seems to be coordinately regulated with production of siderophore (23). *Vibrio anguillarum* also expresses at least two new outer membrane proteins in low-iron conditions (6).

In these studies we examined the ability of several clinical and environmental isolates of *V. cholerae* to produce and utilize siderophores in iron-limited medium. Additionally, we observed several proteins in the outer membranes of iron-starved cells which were absent when cells were grown with sufficient iron.

MATERIALS AND METHODS

Bacterial strains and growth of cultures. CA401, CA385, and CA412 are classical *V. cholerae* strains isolated from patients (10, 31). Lou15 and 7990 are El Tor strains of *V. cholerae* isolated from patients from different outbreaks (31). V69 is an El Tor isolate from Chesapeake Bay (15). All strains were maintained frozen at -70°C in brain heart infusion broth containing 15% glycerol. Working stocks of strains were obtained by culturing the freezer stock on meat extract agar. The Tris-buffered medium (T medium) of Simon and Tessman (32) without added iron was supplemented with 0.4% sucrose and used as iron-poor medium. The merits of this medium for production of siderophores have been described previously (22, 24). Iron in the form of ferrous sulfate was added where indicated. Cultures were grown at 37°C with shaking, and growth was measured turbidometrically with a Klett colorimeter using a no. 54 filter. Cells were passaged twice in T

medium to deplete the iron stores resulting from cultivation on rich medium. The second passage was used in all studies.

Chemicals. Brain heart infusion broth, peptone, and meat extract were obtained from Difco Laboratories, Detroit, Mich. Trizma base (Tris) for T medium, ethylenediamine-di-(*o*-hydroxyphenyl acetic acid) (EDDA), $\text{FeSO}_4 \cdot 7 \text{H}_2\text{O}$, DNase I, brilliant blue R (Coomassie Blue), and sodium lauryl sulfate (SDS) were purchased from Sigma Chemical Co., St. Louis, Mo. Acrylamide and bisacrylamide were obtained from Bio-Rad Laboratories, Richmond, Calif. Tris (ultrapure) for electrophoresis was purchased from Schwarz/Mann, Orangeburg, N.Y. The following protein standards were purchased from Pharmacia Fine Chemicals, Piscataway, N.J.: ferritin half-unit, 220 kilodaltons (220K); phosphorylase *b*, 94K; albumin, 67K; catalase, 60K; ovalbumin, 43K; lactate dehydrogenase, 36K; and carbonic anhydrase, 30K. $\text{H}_2^{35}\text{SO}_4$ was obtained from New England Nuclear Corp., Boston, Mass. All other chemicals were reagent grade.

Detection of siderophores. The method of Atkin et al. (3) was used to detect hydroxamic acids. Production of phenolates was demonstrated by the method of Arnow (2).

Cross-feeding studies (utilization of siderophore). Cross-feeding studies were performed essentially as described previously (24). Luria broth agar (pH 7.4) containing various concentrations of deferrated EDDA (29), an iron chelator, was stored at 4°C for 24 h before use. The agar was remelted, and 10^3 bacteria per ml were added. The inoculated agar was poured into petri dishes, and 5 μl of overnight Luria broth cultures was spotted on the surface of the hardened agar; 10 μl of 2.7 mM 2,3-dihydroxybenzoic acid (DBA) or 1 mM enterochelin was spotted on disks on the surface. Zones of stimulation were measured after 24 h of incubation at 37°C. DBA and enterochelin were provided by C. F. Earhart.

SDS-PAGE of total proteins from cells. Stationary phase cells were solubilized in Laemmli solubilization buffer (17), and proteins were separated by the discontinuous SDS-polyacrylamide gel electrophoresis (PAGE) system of Ames (1). The slab gels, which were 0.7 mm thick and consisted of a 5% stacking gel and a 10% separating gel, were run at 30 mA until the tracking dye was 1 to 2 cm from the bottom (approximately 3 h). The gels were stained with brilliant blue-R (Coomassie blue) (17), destained, and dried.

Preparation of outer membranes. Cultures of mid-log phase cells were harvested by centrifugation at 6,000 $\times g$ for 10 min at 4°C. The pellets were frozen at -70°C for at least 24 h. Pellets were suspended in 10 mM sodium phosphate buffer (pH 7) with 5 mM MgSO_4 . Flagella were removed by shearing in an omnimixer (at setting of 5) at 4°C for 45 s. Total cell envelopes were prepared by the sonic disruption of cells by the procedure of Inouye and Guthrie (13) with the addition of 10 μg of DNase per ml before sonication. Outer membranes were prepared by the method of Filip et al. (8) by using 0.5% Sarkosyl for 20 min at room temperature to solubilize the inner membrane. Protein concentrations were determined by the method of Lowry et al. (19). Protein solubilization and SDS-PAGE were as described for total cell proteins.

Inner membrane contamination of outer membrane preparations was determined by the method of Matsu-

shita et al. (20) as modified by Kelley and Parker (16). Outer membranes and total envelope preparations (0.6 to 0.8 mg of protein per ml) were suspended in 60 mM sodium phosphate buffer (pH 7.2). The difference in absorbance (550 to 575 nm) between a dithionite-reduced sample and an oxidized sample was used to calculate the cytochrome *b* content.

Kinetics of appearance of iron-induced proteins. $\text{H}_2^{35}\text{SO}_4$ (5 $\mu\text{Ci/ml}$) was added to log phase cells in T medium without Na_2SO_4 . One hour later deferrated EDDA (29) was added (250 $\mu\text{g/ml}$ for Lou15 and 50 $\mu\text{g/ml}$ for CA401), and cells were harvested at various times. Outer membranes were isolated, and SDS-PAGE was performed as above. Autoradiograms were prepared by using Kodak safety film NS (Eastman Kodak Co., Rochester, N.Y.).

Immunoprecipitation of cell surface proteins. Young adult New Zealand white rabbits were immunized with 10^9 formalinized cells that had been grown in T medium without added iron. Four weeks later the animals were boosted with 10^9 live cells; 3 weeks later they were boosted again. *Vibrio* agglutinins were detected as early as 2 weeks after the first boost. The immunoprecipitation was essentially that of Hansen et al. (11), with cells labeled with $^{35}\text{SO}_4$ to mid-log phase in T medium. The incubation of whole cells with heat-inactivated antisera, lysis of cells, coprecipitation of immune complexes with Cowan I strain of *Staphylococcus aureus* (protein A), SDS-PAGE, and autoradiography were identical to Hansen's procedures.

RESULTS

Siderophore synthesis and utilization. Fifteen clinical and environmental *V. cholerae* isolates were tested for production of phenolates and hydroxamates, the two principal classes of microbial siderophores. All *V. cholerae* tested produced a phenolate siderophore as determined by the Arnow reaction (2) when grown in T medium without added iron. None of the strains produced detectable hydroxamate-type siderophore. The ratio of absorbancy at 515 nm of the Arnow reaction per 10^9 cells was used to compare the relative amounts of siderophore synthesized by these strains. All ratios were determined by using stationary phase cultures since siderophore concentration is maximal at this stage of growth. The ratio for CA401, a classical strain isolated from a patient, was 0.06 ± 0.005 (standard error of the mean). Two other classical strains from the same outbreak as CA401 (but of a different serotype) also synthesized the siderophore, but grew to lower densities in low-iron medium than did CA401. The ratios for these two strains, CA385 and CA412, were 0.08 and 0.06, respectively, but the stationary phase cell populations were 3 to 5 times lower than that of CA401. Two unrelated El Tor clinical isolates, Lou15 and 7990, grew well in T medium without added iron and produced more siderophore per unit of cells than did CA401; ratios of 0.09 ± 0.006 and 0.08 ± 0.007 , respectively, were obtained. The environmental isolate, V69, grew

less well than the clinical isolates (2 to 4 times fewer cells in the stationary phase population), but it produced 2.5 to 3 times more siderophore per unit of cells than did the other El Tor strains and 4 times more than did CA401 (ratio of 0.23 ± 0.009).

To determine when the phenolate could first be detected in the growth medium, Lou15 was grown to late log phase in T medium plus $3 \mu\text{M}$ added iron and then downshifted by dilution 1:20 into T medium without added iron. Growth continued without a lag, but the siderophore was not detected in the growth medium until 3.5 h after downshift. The phenolate continued to accumulate in the medium after the cells entered stationary phase.

To look for production and utilization of siderophores, we examined the ability of a classical strain (CA401), and El Tor strain (Lou15), and an environmental El Tor isolate (V69) to stimulate growth of lawns of themselves and each other in Luria agar containing different concentrations of the iron chelator, EDDA, which starves the cells for iron (Table 1). Growth of the lawn is stimulated only around sites of active siderophore synthesis. All of the strains stimulated moderate to very good growth of all the lawns at $250 \mu\text{g}$ of EDDA per ml. CA401 was the least effective feeder; at the highest concentration of EDDA ($750 \mu\text{g}/\text{ml}$) it stimulated only sparse growth of Lou15 and V69 lawns and no growth of a CA401 lawn. Lou15 stimulated the best growth at the higher concentrations of EDDA. At $750 \mu\text{g}$ of EDDA per ml, Lou15 stimulated good growth, whereas V69 and CA401 stimulated sparse or no growth of the lawns.

Enterochelin and DBA, an intermediate in the biosynthesis of enterochelin and other phenolates (18), were also examined for their ability to stimulate growth (Table 1). DBA (2.7 mM) stimulated good growth of a Lou15 lawn at 48 h and

good growth of a V 69 lawn at 24 h, but only at the lowest concentration of EDDA. In contrast, growth of CA401 was not stimulated by DBA. Enterochelin was more effective than DBA, stimulating growth comparable to that obtained with the classical strain CA401. However, growth stimulation by enterochelin or the enterochelin-producing strain *S. typhimurium* LT2 (data not shown) was less than that produced by the El Tor strain Lou15.

Effect of iron limitation on cell envelope proteins. The effect of iron limitation on cell proteins was studied by using Lou15 cells harvested at various times during growth in low-iron medium. SDS-PAGE of total cell proteins revealed a large, approximately 220K protein as early as 3.5 h after downshift from medium containing added iron (Fig. 1), corresponding to the time when the phenolate was first detected in the growth medium. This high-molecular-weight protein was present in preparations of total proteins from all 15 strains grown without added iron. Another change seen with iron starvation was the increase in amount of the protein with apparent M_r of 39K (Fig. 1). However, the amount of the 39K protein may be related to the growth phase of the cells, as some increase of this protein with time was also noted when cells were grown in the presence of iron. Additionally, in some of the preparations there was the suggestion of appearance of new proteins in the 60K to 80K range in the iron-starved cells.

To determine the location of the new proteins, we fractionated whole cells into subcellular components: total cell envelope, inner membrane, outer membrane, and cytosol. Outer membranes were separated from inner membranes by treatment with Sarcosyl, a detergent which has been shown to selectively solubilize the inner membranes of *E. coli* (8) and *V. anguillarum* (6). Outer membranes of *V. cholerae* obtained by this method had SDS-PAGE

TABLE 1. Production and utilization of siderophores by *V. cholerae*

Indicator strain ^a	EDDA concn ($\mu\text{g}/\text{ml}$)	Growth ^b stimulated by:				
		Lou15	CA401	V69	Enterochelin (1.0 mM)	DBA (2.7 mM)
CA401	250	++	++	+++	+	—
	500	++	±	++	±	—
	750	+	—	±	—	—
Lou15	250	+++	++	+++	++	± ^c
	500	++	±	+	±	—
	750	++	±	±	—	—
V69	250	+++	++	+++	++	+
	500	++	±	+	±	—
	750	+	±	±	—	—

^a Lawns were seeded with 10^3 indicator bacteria per ml.

^b Amount of growth (diameters of zones of stimulation) at 24 h: +++, $\geq 30 \text{ mm}$; ++, 20 to 29 mm; +, 15 to 19 mm; ±, 10 to 14 mm (tiny colonies); —, no zone.

^c Good growth was detected at 48 h.

FIG. 1. SDS-PAGE of Lou15 total proteins after downshift from T medium plus $3 \mu\text{M}$ FeSO_4 to T medium without added iron. Protein standards with indicated M_r values (K) are shown at left. Arrows point to the approximately 220K protein and the 39K protein.

profiles (Fig. 2) similar to those of outer membranes prepared by the density gradient centrifugation and Triton X-100 extraction method of Kelley and Parker (16). Cytochrome scans indicated less than 5% inner membrane contamination of these outer membrane preparations.

SDS-PAGE of outer membrane preparations (10 to 30 μg of protein per well) of iron-starved cells of Lou15 revealed the presence of several proteins in the 73K to 77K range and a 23K protein which were present in only trace amounts in cells grown in sufficient iron (Fig. 2). These cells were starved for iron by the addition of the iron chelator EDDA to exponentially growing cells, thus allowing the kinetics of appearance of these proteins to be determined. The iron-regulated proteins appeared in the outer membrane as early as 30 min after the addition of EDDA to cells growing in T medium plus $10 \mu\text{M}$ added iron (Fig. 2). It was not possible to correlate the time of appearance of these proteins with the synthesis of the siderophore since the presence of EDDA in the medium interfered with the Arnow reaction.

The amount of protein on the gels was reduced to facilitate separation of the iron-regulated proteins (Fig. 3). Under these conditions, five proteins were found in the outer membrane of the iron-starved cells, but not in the cells grown in sufficient iron. The proteins have apparent M_r values of 62K, 73K, 75K, 76K, and 77K. Identical results were obtained with the classical strain CA401. V69 also expressed these proteins under conditions of iron limitation, and the proteins were present in the outer membrane of this environmental isolate even when the cells were grown with concentrations of iron ($10 \mu\text{M}$) that repressed their synthesis in Lou15 and CA401.

The 220K protein was also detected at 30 min after the addition of EDDA to the culture, although its intensity in the outer membrane was much lower than the other iron-regulated proteins. This protein could be seen more readily in the total envelope or in the Sarkosyl-solubilized inner membrane preparations (data not shown).

To determine which, if any, of the outer membrane proteins were heat modifiable or pep-

FIG. 2. Autoradiogram of $\text{H}_2^{35}\text{SO}_4$ -labeled outer membrane proteins of Lou15 after the addition of the iron chelator EDDA to cells growing in T medium plus $10\ \mu\text{M}\ \text{FeSO}_4$. Lanes: (a) no EDDA; and (b) 30 min, (c) 90 min, (d) 3 h, and (e) 6 h after addition of EDDA.

tidoglycan associated, the preparations were solubilized in Laemmli solubilization buffer at 25, 37, 60, or 100°C (7, 27) (Fig. 4). Four of the proteins were solubilized, although in smaller quantities, at 25 and 37°C , suggesting that they are neither heat modifiable nor peptidoglycan associated. The 62K protein and the 40K major outer membrane protein (MOMP) were only seen when the solubilization temperature was 60°C or higher, suggesting that they are peptidoglycan associated.

Immunoprecipitation of cell surface components. Since the 220K protein appeared in the Sarkosyl-soluble fraction in some preparations and in the Sarkosyl-insoluble fraction in others, we wanted to determine whether it was an outer membrane protein exposed on the cell surface. Rabbit antiserum directed against whole Lou15 cells grown in T medium without added iron was prepared. The antiserum was incubated with Lou15 cells grown with or without added iron, the cells were lysed with detergents and EDTA, and the cell surface antigen-antibody complex

FIG. 3. SDS-PAGE of outer membrane proteins of Lou15. Lanes: (a) protein standards with indicated molecular masses (K); (b) outer membrane proteins of Lou15 grown in T medium without added iron; and (c) outer membrane proteins of Lou15 grown in T medium plus $10\ \mu\text{M}$ added FeSO_4 .

was coprecipitated with *S. aureus* protein A. A number of proteins, including the MOMP, were precipitated with this antiserum (Fig. 5). The 220K protein was also immunoprecipitated, suggesting that it is a cell surface protein. This protein was only seen in cells grown without added iron (Fig. 5, arrow). Three other proteins (92K, 89K, and 23K; arrows) were seen only on the surface of cells grown without added iron. Additionally, some proteins are found in much higher concentrations on the surface of cells grown in the presence of iron. The most obvious of these are a 97K doublet and bands of 77K and 21K (arrows). The relative amounts of the 97K and the 77K protein immunoprecipitated depend on the antiserum used, and differences among individual rabbits were noted.

FIG. 4. SDS-PAGE of outer membrane proteins of Lou15 solubilized in Laemmli solubilization buffer at the following temperatures: (a) 25°C, (b) 37°C, (c) 60°C, and (d) 100°C. Arrows point to the peptidoglycan-associated proteins.

DISCUSSION

High-affinity iron transport systems are routinely expressed by *V. cholerae* during growth in low-iron media, and all of the *V. cholerae* strains tested produced a phenolate-type siderophore. There were, however, differences among strains in production and utilization of siderophores.

One way to measure these differences is to look at both the density of growth in low-iron medium and the amount of siderophore produced. CA401 grew well in T medium without added iron and produced a moderate amount of siderophore. Two other classical strains grew poorly in T medium, however. Two El Tor clinical isolates, Lou15 and 7990, grew well in low-iron medium and produced more siderophore than any of the classical isolates. The environmental El Tor strain, V69, grew less well than CA401, Lou15, or 7990 in T medium. However, V69 produced the most siderophore per unit of cells and continued to synthesize the siderophore at iron concentrations that repressed synthesis in the other strains. This suggests that V69 needs more iron than the clinical isolates, is less efficient at transporting the iron-

FIG. 5. Autoradiogram of immunoprecipitated $\text{H}_2^{35}\text{SO}_4$ -labeled cell surface proteins. Lanes: (a) cells grown without added iron; (b) cells grown with 20 μM FeSO_4 . Arrows point to differences in the banding patterns.

siderophore complex, or possesses an iron-scavenging system that is less tightly controlled by iron.

Another method for examining synthesis and utilization of siderophore is to look at the ability of the strains to stimulate growth of bacterial lawns in medium containing a strong iron chelator. CA401 was the least efficient growth stimulator. It stimulated slight growth of V69 and Lou15 and no growth of a CA401 lawn at the highest concentration of EDDA. Lawns of CA401 were stimulated more effectively by Lou15 and V69 than by itself. This suggests that CA401 synthesizes less siderophore than the El Tor strains. Furthermore, there was no stimulation of growth of a CA401 lawn at the concentration of DBA used in these studies. This suggests that CA401 uses DBA less efficiently than does V69 or Lou15. Lou15 was the most effective of the three strains in reversing the growth inhibiting effects of EDDA. Lou15 stimulated growth of lawns at EDDA concentrations that stifled the ability of the other strains to crossfeed. V69 stimulated excellent growth of lawns at the lowest concentration of EDDA, but was much less effective at feeding lawns at higher EDDA

concentrations. Since it was shown by the Arnow test to produce more siderophore in T medium than Lou15, it is possible that V69 synthesizes a different siderophore which has a lower affinity for iron than does the Lou15 siderophore.

The fact that every *V. cholerae* strain tested so far expressed a large protein (approximately 220K) under conditions of iron starvation suggests that either this protein is involved with the high-affinity iron transport system (25), or it is regulated by the same elements that control this transport system. The 220K protein was found in the cells at the same time the siderophore could first be detected in the cultures. This large protein appeared to be loosely associated with the outer membrane. It was seen in total envelope preparations, but was extracted by 0.5% Sarkosyl treatment, whereas most of the other outer membrane proteins were resistant to this treatment. It is apparently a cell surface component, since it was immunoprecipitated with antisera directed against the cell surface of iron-starved cells.

The appearance in the outer membrane of iron-starved cells of five proteins with molecular weights in the range of those expressed by iron starved *E. coli* (4) suggests that one or more of them might be receptors for the iron-siderophore complex. These proteins appeared to be synthesized and exported to the outer membrane as early as 30 min after iron deprivation induced by the addition of EDDA.

It appears that the 40K MOMP and the 62K protein are peptidoglycan associated, since they are solubilized only at 60°C or above. In *E. coli* many peptidoglycan associated proteins are porins (7). Possibly both the MOMP and the 62K protein function as pores.

Kabir has previously indicated that the MOMP of *V. cholerae* is heat modifiable (14), but reports an M_r of 48K rather than 40K for this protein. Other studies on *V. cholerae* outer membranes report that the molecular weight of the MOMP is 45K (16) or 37.6K (G. M. Resnick, personal communication). These differences could reflect differences in growth conditions, membrane preparation, gel electrophoresis conditions, or protein solubilization methods.

The immunoprecipitation studies show that there are differences in the surface antigens of cells grown with and without added iron. Although the 220K protein and the MOMP are present, the rest of the band pattern found in the autoradiogram of immunoprecipitated surface antigens differs from the pattern of outer membrane protein preparations. No unique bands in the 62K to 77K range are present on the autoradiograms of cell surface components from iron-starved cells. However, a band at 94K and one

at 89K are present in these preparations and absent in the preparations of cells grown with sufficient iron. Differences in electrophoretic pattern of immunoprecipitated cell surface proteins and solubilized outer membrane proteins may result from differences in solubilization procedures and from the fact that some of the outer membrane proteins might be buried inside the bilayer and may not be readily accessible to antibodies. Surface components are of interest because they are the first thing a host "sees" when it encounters a foreign entity. Host responses to these antigens could be a first line of defense through opsonization, complement-mediated cell lysis, or other antibody-directed mechanisms of bacterial stasis or destruction.

These studies demonstrate the responses of *V. cholerae* strains to iron limitation. Clinical isolates of *V. cholerae* produce a high-efficiency iron-chelating system when grown in low-iron medium. However, *V. cholerae* is not an invasive pathogen and remains on the gut mucosal surface where the concentration of iron may be sufficient to repress the high-efficiency iron-scavenging system. Therefore it will be necessary to determine which, if any, of the iron-regulated proteins are expressed during growth in vivo in a suitable animal model. This will help elucidate the possible role for a high-efficiency iron transport system in colonization of and disease production by an epithelial surface pathogen like *V. cholerae*. Additionally, salt and brackish water environments are likely to be limited in soluble iron. A *V. cholerae* isolate from such an environment produces a high-efficiency iron transporting system which continues to be expressed at iron levels that repress expression in clinical isolates. Furthermore, there appear to be differences in utilization of siderophore by clinical and environmental isolates of *V. cholerae*.

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LITERATURE CITED

1. Ames, G. F.-L. 1974. Resolution of bacterial proteins by polyacrylamide gel electrophoresis on slabs. *J. Biol. Chem.* **249**:634-644.
2. Arnow, L. E. 1937. Colorimetric determination of the components of 3,4-dihydroxyphenylalanine-tyrosine mixtures. *J. Biol. Chem.* **228**:531-537.
3. Atkin, C. L., J. B. Nellands, and J. H. Pfaff. 1970. Rhodotorulic acid from species of *Leucosporidium*, *Rho-*

- dosporidium*, *Rhodotorula*, *Sporidiobolus*, and *Sporobolomyces*, and a new alanine containing ferrichrome from *Cryptococcus melibiosum*. J. Bacteriol. 103:722-733.
4. Braun, V., R. E. W. Hancock, K. Hantke, and A. Hartman. 1976. Functional organization of the outer membrane of *Escherichia coli*: phage and colicin receptors as components of iron uptake systems. J. Supramol. Struct. 5:37-58.
 5. Brot, N., J. Goodwin, and H. Fales. 1966. In vivo and in vitro formation of 2,3-dihydroxybenzoylserine by *Escherichia coli* K-12. Biochem. Biophys. Res. Commun. 25:454-461.
 6. Crosa, J. H., and L. L. Hodges. 1981. Outer membrane proteins induced under conditions of iron limitation in the marine fish pathogen *Vibrio anguillarum*. Infect. Immun. 31:223-227.
 7. DiRienzo, J. M., K. Nakamura, and M. Inouye. 1978. The outer membrane proteins of gram-negative bacteria: biosynthesis, assembly, and functions. Annu. Rev. Biochem. 47:481-532.
 8. Filip, C., G. Fletcher, J. L. Wulff, and C. F. Earhart. 1973. Solubilization of the cytoplasmic membrane of *Escherichia coli* by the ionic detergent sodium-lauryl sarcosinate. J. Bacteriol. 115:717-722.
 9. Finkelstein, R. A. 1973. Cholera. Crit. Rev. Microbiol. 2:553-623.
 10. Gardner, E. W., S. T. Lyles, C. E. Lankford, and S. J. Hagens. 1963. *Vibrio cholerae* infection in the embryonated egg. J. Infect. Dis. 112:264-272.
 11. Hansen, E. J., C. F. Frisch, and K. H. Johnston. 1981. Detection of antibody-accessible proteins on the cell surface of *Haemophilus influenzae* type B. Infect. Immun. 33:950-953.
 12. Hollifield, W. C., Jr., and J. B. Neillands. 1978. Ferric enterobactin transport system in *Escherichia coli* K12. Extraction, assay, and specificity of the outer membrane receptor. Biochemistry 17:1922-1928.
 13. Inouye, M., and J. P. Guthrie. 1969. A mutant which changes a membrane protein of *E. coli*. Proc. Natl. Acad. Sci. U.S.A. 64:957-961.
 14. Kabir, S. 1980. Composition and immunochemical properties of outer membrane proteins of *Vibrio cholerae*. J. Bacteriol. 144:382-389.
 15. Kaper, J., H. Lockman, R. R. Colwell, and S. W. Joseph. 1979. Ecology, serology, and enterotoxin production of *Vibrio cholerae* in Chesapeake Bay. Appl. Environ. Microbiol. 37:91-103.
 16. Kelley, J. T., and C. D. Parker. 1981. Identification and preliminary characterization of *Vibrio cholerae* outer membrane proteins. J. Bacteriol. 145:1018-1024.
 17. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227:680-685.
 18. Lankford, C. E. 1973. Bacterial assimilation of iron. Crit. Rev. Microbiol. 2:273-331.
 19. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.
 20. Matsushita, K., O. Adachi, E. Shinagawa, and M. Ameyama. 1978. Isolation and characterization of outer and inner membranes from *Pseudomonas aeruginosa* and effect of EDTA on the membranes. J. Biochem. (Tokyo) 83:171-181.
 21. McIntosh, M. A., S. S. Chenault, and C. F. Earhart. 1979. Genetic and physiological studies on the relationship between colicin B resistance and ferrienterochelin uptake in *Escherichia coli* K-12. J. Bacteriol. 137:653-657.
 22. McIntosh, M. A., and C. F. Earhart. 1976. Effect of iron on the relative abundance of two large polypeptides of the *Escherichia coli* outer membrane. Biochem. Biophys. Res. Commun. 70:315-322.
 23. McIntosh, M. A., and C. F. Earhart. 1977. Coordinate regulation by iron of the synthesis of phenolate compounds and three outer membrane proteins in *Escherichia coli*. J. Bacteriol. 131:331-339.
 24. Payne, S. M. 1980. Synthesis and utilization of siderophores by *Shigella flexneri*. J. Bacteriol. 143:1420-1424.
 25. Payne, S. M., and R. A. Finkelstein. 1978. Siderophore production by *Vibrio cholerae*. Infect. Immun. 20:310-311.
 26. Perry, R. D., and C. L. San Clemente. 1979. Siderophore synthesis in *Klebsiella pneumoniae* and *Shigella sonnei* during iron deficiency. J. Bacteriol. 140:1129-1132.
 27. Pollack, J. R., and J. B. Neillands. 1970. Enterobactin, an iron transport compound from *Salmonella typhimurium*. Biochem. Biophys. Res. Commun. 38:989-992.
 28. Pugaley, A. P., and P. Reeves. 1977. The role of colicin receptors in the uptake of ferrienterochelin by *Escherichia coli* K-12. Biochem. Biophys. Res. Commun. 74:903-911.
 29. Rogers, H. J. 1973. Iron-binding catechols and virulence in *Escherichia coli*. Infect. Immun. 7:445-456.
 30. Rosenbusch, J. P. 1974. Characterization of the major envelope protein from *Escherichia coli*. Regular arrangement on the peptidoglycan and unusual dodecyl sulfate binding. J. Biol. Chem. 249:8019-8029.
 31. Sigel, S. P., S. Lanier, V. S. Baselski, and C. D. Parker. 1980. In vivo evaluation of pathogenicity of clinical and environmental isolates of *Vibrio cholerae*. Infect. Immun. 28:681-687.
 32. Simon, E. H., and I. Tessman. 1963. Thymidine-requiring mutants of phage T4. Proc. Natl. Acad. Sci. U.S.A. 50:526-532.
 33. Weinberg, E. D. 1978. Iron and infection. Microbiol. Rev. 42:45-66.
 34. Wilkins, T. D., and C. E. Lankford. 1970. Production by *Salmonella typhimurium* of 2,3-dihydroxybenzoylserine and its stimulation of growth in human serum. J. Infect. Dis. 121:129-136.