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Influence of Growth Rate and Iron Limitation on the Expression of Outer Membrane Proteins and Enterobactin by *Klebsiella pneumoniae* Grown in Continuous Culture

JULIA M. T. LODGE, PAUL WILLIAMS,† AND MICHAEL R. W. BROWN*

Microbiology Research Group, Department of Pharmaceutical Sciences, Aston University, Birmingham B4 7ET, United Kingdom

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The influence of the growth rate on outer membrane protein composition and enterobactin production was studied with *Klebsiella pneumoniae* grown under conditions of iron limitation in chemostats. More enterobactin was produced at fast ($D = 0.4\ h^{-1}$) and slow ($D = 0.1\ h^{-1}$) growth rates in continuous cultures than in either logarithmic- or stationary-phase batch cultures. When the growth rate was controlled under conditions of carbon limitation and the iron level was reduced to 0.5 μM, the iron-regulated outer membrane proteins and enterobactin were induced at the fast growth rate. At the slow growth rate, although the iron-regulated outer membrane proteins were barely visible, a significant level of enterobactin was still produced. These results suggest that under conditions of either carbon or iron limitation, the growth rate can influence the induction of the high-affinity iron uptake system of *K. pneumoniae*. Other outer membrane proteins, including a 39-kilodalton peptidoglycan-associated protein, were found to vary with the growth rate and nutrient limitation.

Iron is an important microbial nutrient which is often not readily available to microorganisms. In aerobic environments at a neutral pH, it predominates as highly insoluble ferric hydroxide polymers (7). During many infections, the withholding of iron by transferrin and lactoferrin constitutes an important component of nonspecific mammalian defense mechanisms (13, 17, 39, 40). The ability to obtain iron is therefore an important virulence factor and, in response to iron deprivation, many microorganisms produce low-molecular-weight iron-chelating agents (or siderophores) and membrane receptor proteins (17, 31, 32, 39, 40). These high-affinity iron uptake systems are expressed by some bacteria growing in vivo during infections in both animals (18, 35) and humans (5, 24, 36). We recently showed that *Klebsiella pneumoniae* grown in batch cultures in iron-depleted media or in serum induced at least six new iron-regulated outer membrane (OM) proteins (IRMPs) together with the iron chelator enterobactin (enterochelin) (41).

During infections, bacterial growth rates appear to be slow (4, 11, 37), and nutrients as well as or other than iron may be growth limiting (6, 37, 39). Chemostat culturing offers many advantages over batch culturing in the study of microbial responses to varying growth rates. The use of chemostat cultures enables either the specific growth rate to be varied by changing the dilution rate while maintaining the cells in a constant physical and chemical environment or a constant specific growth rate to be maintained while physiochemical parameters of growth, such as pH, temperature, or nutrient availability, are varied (10, 16, 21). We previously showed that relatively low iron concentrations in batch cultures can cause envelope changes without altering the growth rate (41). In this paper we describe the influence in chemostats of the growth rate controlled either by iron or carbon limitation on the OM protein profile and on the production of the siderophore enterobactin by *K. pneumoniae*. In the carbon-limited chemostat, the medium iron content was lowered to concentrations close to but above the concentration which would be rate limiting.

* Corresponding author.
† Present address: Department of Pharmacy, Nottingham University, Nottingham, United Kingdom.

MATERIALS AND METHODS

Organisms and growth conditions. *K. pneumoniae* DL1, an encapsulated clinical isolate (41), was grown aerobically in continuous cultures at 37°C in glass chemostats at a working volume of 50 ml maintained at a dilution rate of either $D = 0.1\ h^{-1}$ (doubling time, 6.9 h) or $D = 0.4\ h^{-1}$ (doubling time, 1.7 h). For each experiment, the chemostat was allowed to equilibrate by passing five complete changes of medium through the vessel. Bacteria were also grown to the stationary phase in batch cultures. The simple salts medium (SSM) used for all cultures was that described by Williams et al. (41). This medium was designed such that each essential nutrient was present in a sufficient excess to enable exponential growth in a batch culture to an A_{470} of 10.0 before the onset of the stationary phase. This permitted the production of cultures depleted of any specific essential nutrient, all other nutrients being present in excess. For carbon limitation, the glucose concentration was reduced to 3.5 mM, which was sufficient to support growth to an A_{470} of 1.0 at $D = 0.1\ h^{-1}$. For iron limitation, the level of iron was reduced to 0.5 μM by treatment of the phosphate buffer with Chelex resin as described previously (41). This iron-limited SSM supported growth in a chemostat culture to an A_{470} of 2.5 at $D = 0.1\ h^{-1}$. In some experiments, the growth rate was controlled by carbon limitation and the iron level was reduced to 1.5 or 0.5 μM. The same media were also used for batch culture experiments. Glassware was rendered iron free by treatments described previously (41).

Preparation and electrophoresis of OMs. Bacteria were collected on ice from the chemostat overflow, harvested by centrifugation, broken by sonication, and treated with 2% (wt/vol) *N*-lauroylsarcosine (Sigma Chemical Co., St. Louis, Mo.) to prepare OMs as described by Filip et al. (12).

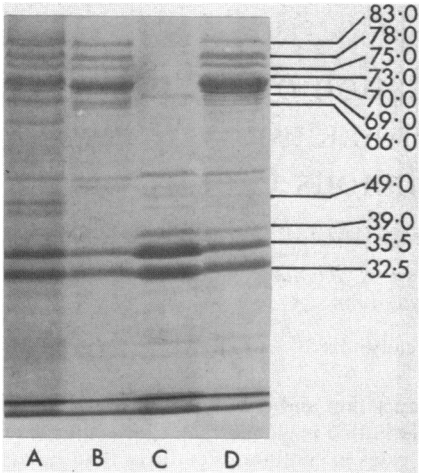

FIG. 1. SDS-PAGE of the OM proteins of *K. pneumoniae* after growth in an iron-limited chemostat culture at a slow growth rate (D = 0.1 h^{-1}) (lane A), in an iron-limited chemostat culture at a fast growth rate (D = 0.4 h^{-1}) (lane B), in an iron-sufficient (20 μM) batch culture (lane C), and in an iron-depleted (0.5 μM) batch culture (lane D). Protein sizes are given in kilodaltons.

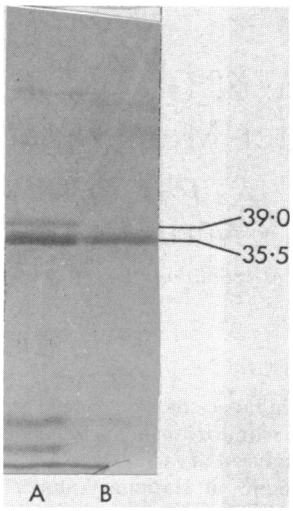

FIG. 2. SDS-PAGE of the peptidoglycan-associated OM proteins of *K. pneumoniae* after growth in an iron-depleted batch culture (lane A) and in a carbon-limited, low-iron chemostat culture at the slow growth rate (lane B). Protein sizes are given in kilodaltons.

Equivalent OM concentrations were achieved by resuspension of bacterial cells to the same A_{470} before preparation of OMs. OM samples (50 μl) were loaded onto each well. The OM proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Lugtenberg et al. (26) with specially purified sodium dodecyl sulfate (BDH, Poole, England) (1). Gels were fixed, stained with Coomassie brilliant blue, and photographed. Molecular masses were determined relative to phosphorylase *b* (94 kilodaltons [kDa]), bovine serum albumin (68 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), and soybean trypsin inhibitor (20.1 kDa).

Noncovalent peptidoglycan-associated proteins. Bacteria were suspended in 10 mM sodium phosphate buffer (pH 7.2) and sonicated, and the proteins noncovalently bound to peptidoglycan were isolated by the method of Mizuno and Kagayama (29).

Enterobactin estimation. Enterobactin and its degradation products were extracted in ethyl acetate and detected by paper chromatography with a formic acid-ammonium formate solvent as described by Perry and San Clemente (34). Five spots with R_f values of 0.22, 0.45, 0.59, 0.69, and 0.82, which correspond to those described previously for *K. pneumoniae* (34), were observed. Enterobactin and related phenolates were assayed quantitatively by the method of Arnow (2).

Iron estimation. The medium iron content was measured by flameless atomic absorption spectrophotometry with a Perkin-Elmer model 360 spectrophotometer fitted with a deuterium background corrector.

RESULTS AND DISCUSSION

The OMs of *K. pneumoniae* grown in SSM in batch cultures had three OM proteins of 32.5, 35.5, and 39 kDa (Fig. 1, lanes C and D). In iron-depleted SSM, at least six IRMPs of 66 to 83 kDa (Fig. 1, lane D) were present and were repressed after growth in iron-sufficient SSM (Fig. 1, lane C). The 83-kDa protein has recently been proposed to be the enterobactin receptor protein for *K. pneumoniae* (8). The 35.5- and 39-kDa proteins were noncovalently associated with peptidoglycan (Fig. 2, lane A) and are probably related to the OmpF and OmpC porin proteins of *Escherichia coli* K-12, with which they cross-react immunologically (22, 25, 27, 41). The effect of growth in iron-limited chemostats at both slow (D = 0.1 h^{-1}) and fast (D = 0.4 h^{-1}) growth rates on the OM protein profile is shown in Fig. 1, lanes A and B. The protein profiles were closely related, although the 39-kDa peptidoglycan-associated protein present in the batch cultures was absent at both growth rates in the iron-limited chemostat cultures. The OM proteins OmpF and OmpC in *E. coli* K-12 were also affected by changes in growth conditions, in particular, changes in temperature, medium composition, and osmolarity (19, 27, 33). The changes in *E. coli* were such that a constant overall amount of porin protein was observed. However, the 35.5-kDa protein did not appear to be overproduced after repression of the 39-kDa protein. In chemostat or batch cultures containing 0.5 μM Fe^{3+}, more enterobactin was produced at both growth rates in continuous cultures than during the stationary phase in batch cultures (Table 1). The amount of enterobactin produced in continuous cultures was also greater than that produced throughout the growth phase in batch cultures, in which enterobactin concentrations in-

TABLE 1. Influence of the medium iron content on enterobactin production by *K. pneumoniae* in batch and chemostat cultures

SSM carbon content (mM)	SSM iron content (μM)[a]	Enterobactin production (μg/mg of cell dry wt) in:		
		Batch cultures (stationary phase)[b]	Chemostat cultures[d]	
			Slow	Fast
35	20	ND[c]	—	—
	0.5	18.19 ± 2.0	28.25 ± 3.0	35.2 ± 6.4
3.5	20	ND	ND	ND
	0.5	14.1 ± 6.5	11.8 ± 2.1	29.6 ± 0.9

[a] SSM with 1.5 μM iron yielded no detectable enterobactin in any system.
[b] Enterobactin concentrations were determined 10 h after the onset of the stationary phase.
[c] ND, Not detectable.
[d] —, Not done.

creased along the growth curve to a maximum of ca. 18 μg/mg of cell dry weight at 10 h after the onset of the stationary phase (41). This difference was perhaps more significant because the assayed batch culture concentration represented a measure of the total accumulated enterobactin, whereas the chemostat culture concentration was a measure of the steady-state concentration.

In glucose-limited chemostats, the effect of the growth rate on OM proteins and enterobactin production at different iron concentrations was studied. This was done by varying the iron concentration but at concentrations above those that reduce the growth rate. It is not yet clear whether such conditions can strictly be described as glucose limited. Figure 3 shows the OM protein profiles of *K. pneumoniae* grown at both slow (lanes A, B, and C) and fast (lanes D, E, and F) growth rates at iron concentrations of 20 μM (lanes A and D), 1.5 μM (lanes B and E) , and 0.5 μM (lanes C and F). Carbon limitation of growth resulted in the induction of a 49-kDa non-peptidoglycan-associated protein which may be a doublet (Fig. 3, lanes A to F, and Fig. 2, lane B) and which was only present at low levels in the OMs of cells grown in batch cultures in the same medium (Fig. 4). Other workers have also reported the presence of a similar protein in the OM of *Klebsiella* spp. under conditions of carbon (glucose, glycerol, or maltose) limitation (38). Sterkenberg et al. (38) suggested that this protein may be related to the MalB protein of *E. coli* (30) and the glucose-inducible D1 protein of *Pseudomonas aeruginosa* (20). However, unlike the MalB protein (3), the 49-kDa protein was not noncovalently associated with peptidoglycan. (Fig. 2, lane B).

At the fast growth rate, carbon-limited *K. pneumoniae* expressed both the 35.5- and the 39-kDa peptidoglycan-associated proteins (Fig. 3, lanes D, E, and F), as did batch-cultured cells. However, the 39-kDa protein was repressed at the slow growth rate (Fig. 3, lanes A, B, and C). Therefore, under carbon limitation, the growth rate per se can influence the expression of a peptidoglycan-associated OM protein in *K. pneumoniae*. Extraction of the major peptidoglycan-associated proteins confirmed that only the 35.5-kDa protein remained (Fig. 2, lane B).

Reduction of the SSM iron concentration to about 0.5 μM in the carbon-limited chemostats resulted in the induction of

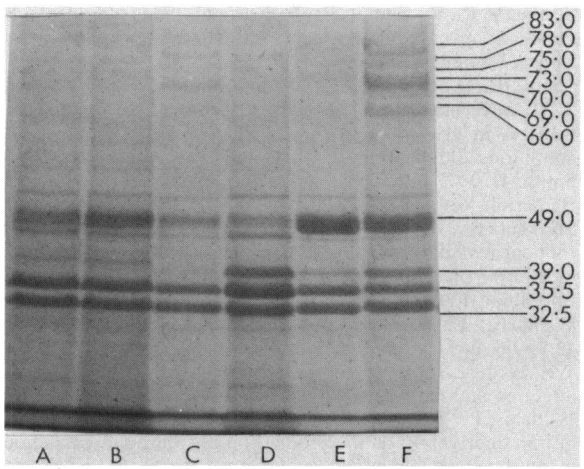

FIG. 3. SDS-PAGE of the OM proteins of *K. pneumoniae* grown in a carbon-limited chemostat culture at slow (lanes A, B, and C) and fast (lanes D, E, and F) growth rates in SSM containing 20 μM Fe^{3+} (lanes A and D), 1.5 μM Fe^{3+} (lanes B and E), and 0.5 μM Fe^{3+} (lanes C and F). Protein sizes are given in kilodaltons.

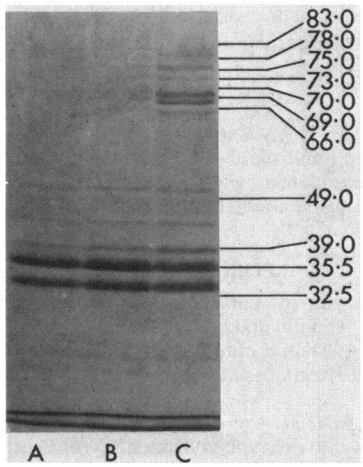

FIG. 4. SDS-PAGE of the OM proteins of *K. pneumoniae* after growth in a batch culture in carbon-depleted SSM containing 20 μM Fe^{3+} (lane A), 1.5 μM Fe^{3+} (lane B), and 0.5 μM Fe^{3+} (lane C). Protein sizes are given in kilodaltons.

the IRMPs to an appreciable extent only at the fast growth rate (Fig. 3, lane F). Enterobactin, however, was produced by the organism at both dilution rates, although at a lower level at the slow growth rate (Table 1). Growth in batch cultures in the same carbon-limited, low-iron medium also resulted in the induction of the IRMPs (Fig. 4, lane C). There was no induction of the IRMPs or production of enterobactin by cells grown in carbon-limited SSM containing greater than 1.5 μM iron in the batch (Fig. 4, lanes A and B) or chemostat (Fig. 3, lanes A, B, D, and E; Table 1) cultures. The production of enterobactin and its 81-kDa OM receptor protein (FepA) by *E. coli* K-12 grown in batch cultures in iron-depleted media or media to which various biological chelators had been added is coordinately controlled (14, 23, 28, 32). Furthermore, mutants which lack this receptor protein are unable to utilize ferric enterobactin (9, 43). The production of enterobactin by slow-growing, carbon-limited *K. pneumoniae* in SSM containing about 0.5 μM Fe^{3+} without induction of the IRMPs implies that the two components of the high-affinity iron uptake system may not always be coordinately controlled. In iron-plentiful SSM, the IRMPs never completely disappear but remain in the OM in amounts just detectable by Coomassie brilliant blue staining of the sodium dodecyl sulfate polyacrylamide gel (41). There may therefore be sufficient levels of the enterobactin receptor protein to ensure that enough ferric enterobactin can enter the cell to enable maintenance of the slow growth rate in continuous cultures. The complexity of the high-affinity iron uptake systems in *Klebsiella* spp. has been further highlighted by recent work with a strain recovered without subculturing from urinary tract infections (24, 36). Although only three IRMPs (73, 75, and 83 kDa) were observed in the OM of the in vivo-grown strain, growth of the same strain in iron-depleted laboratory media resulted in the induction of at least six IRMPs (24, 36), as does the growth of *Klebsiella* spp. in serum (41). Thus, although a slow growth rate under iron limitation in continuous cultures may mimic the in vivo situation more closely than batch cultures, other factors, such as surface growth, temperature, pH, osmolarity, availability of other nutrients, and the presence of host antibodies, may also influence the expression of the high-affinity iron uptake systems. Indeed, growth temperature has been

shown to influence the acquisition of iron by both *Salmonella typhimurium* and *E. coli* (15, 42).

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