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Invasion of Rabbit Ileal Tissue by *Enterobacter cloacae* Varies with the Concentration of OmpX in the Outer Membrane

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The outer membrane protein OmpX of *Enterobacter cloacae* shows high amino acid homology with virulence proteins PagC and Rck from *Salmonella typhimurium* and with Ail from *Yersinia enterocolitica*. Here we demonstrate a role for OmpX in the invasion of rabbit ileal tissue by *E. cloacae*. An organ culture system was used for maintenance of rabbit gut tissue during the experiments. The invasivenesses of three *E. cloacae* strains, which differed in OmpX content, were compared with each other and with that of *Salmonella typhimurium* TML (a highly invasive strain) and *S. typhimurium* LT7 (a noninvasive strain). There was no significant difference between the invasiveness of the wild type and that of an *ompX* deletion mutant strain of *E. cloacae*; they were equally as invasive or less invasive than *S. typhimurium* LT7. The invasiveness of an OmpX overproducer strain of *E. cloacae* was 10-fold higher than that of its immediate parent carrying only the multicopy plasmid, higher than that of *S. typhimurium* LT7, but lower than that of *S. typhimurium* TML. The invasiveness of *E. cloacae* thus varied directly with the level of OmpX in the outer membrane in rabbit ileal enterocytes challenged in situ.

Enterobacter cloacae is normally a harmless gut commensal organism; occasionally, it becomes an opportunistic enteric pathogen. A strain of *E. cloacae* which was resistant to both β-lactams (21) and complement-mediated serum killing was isolated from a patient who died of an *E. cloacae* septicemia while undergoing chemotherapy. This organism has been the subject of intense study aimed at elucidating the mechanistic basis of its antibiotic resistance (19) and resistance to serum killing. In this paper, we describe experiments designed to complete our picture of the pathogenic attributes of this strain and, in particular, to explain the basis of acquired invasiveness. For the latter, we have used a recently described technique (1) for measuring the relative invasiveness of enteric pathogens in structurally and functionally intact gut tissue in vitro. The focus of the work is the properties and role in disease causation of the outer membrane protein OmpX.

Protein OmpX of *E. cloacae* is a 17-kDa outer membrane protein. Cloning of the *ompX* gene into *E. cloacae* and *Escherichia coli* on a multicopy plasmid confers decreased susceptibility to several β-lactams and quinolones. Overproduction of OmpX in *E. cloacae* and *E. coli* leads to a decrease in the amounts of the pore proteins OmpF and OmpC (9, 15, 19). The resulting reduction in permeability of the outer membrane induced by OmpX may be the explanation for reduced antibiotic susceptibility.

The nucleotide sequence of the *ompX* gene was determined, and from the deduced amino acid sequence a topological model for OmpX was postulated (18). This model resembles that proposed for *E. coli* pore proteins like PhoE (11, 14, 22) and OmpA (23) and the *Rhodobacter capsulatus* porin (14, 25). It predicts a barrel-like structure consisting of eight β-strands in the membrane, three short β-turns in the periplasm, and four random-coil loops at the bacterial surface.

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The β-strands of OmpX show a high amino acid homology with those of PagC (16) and Rck (10) from *Salmonella typhimurium*, Ail (13) from *Yersinia enterocolitica*, and Lom (2) from lysogenic phage in *E. coli* (Table 1). These proteins are located in the outer membrane and possess similar structural features. Since it has been demonstrated that the surface loops of these proteins are involved in virulence (3), we decided to investigate further the virulence properties of OmpX. In particular, Ail is known to confer both serum resistance (4) and invasiveness to *Y. enterocolitica*. Studies have already been carried out which show that both resistance to complement-mediated killing and colonization of the mouse digestive tract are mediated by OmpX in our clinical isolate of *E. cloacae* (unpublished data).

In this study, we have investigated the invasion of rabbit ileal enterocytes by *E. cloacae* in a newly developed quantitative invasion assay (1) as a function of the amount of OmpX present in wild-type, OmpX-deficient, and *ompX*-overexpressing strains of this organism.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. cloacae* 2249-1 (19) is a clinical isolate with a single *ompX* gene on the chromosome. This strain produces low amounts of OmpX when grown under laboratory conditions. *E. cloacae* JS101 is an *ompX* deletion mutant constructed by insertional inactivation (20). *S. typhimurium* TML and LT7 (7, 8) are, respectively, highly and poorly invasive for rabbit enterocytes (1).

Plasmid pJS04 (19) is a construct of vector pACYC184 (5) and the *ompX* gene. This plasmid was used for high-level expression of *ompX*. The vector pACYC184 was used as an OmpX-negative control for the pJS04 plasmid. The level of production of OmpX in *E. cloacae* 2249-1 (pACYC184) and in *E. cloacae* 2249-1 (pJS04) has been shown before by sodium dodecyl sulfate-polyacrylamide gel analysis (19). The densitometrically quantified amount of [^{35}S]methionine-labelled

TABLE 1. Virulence proteins structurally related to OmpX

Protein	Origin	Function	β-Sheet homology to OmpX (%)	Reference
Rck	*S. typhimurium*	Resistance to complement-mediated killing	55	10
PagC	*S. typhimurium*	Survival in macrophages	50	16
Ail	*Y. enterocolitica*	Invasion in epithelial cells	60	13
Lom	Lysogenic lambda phage in *E. coli*	Unknown	46	1

OmpX produced by *E. cloacae* JS101(pJS04) was 10-fold higher than that produced by *E. cloacae* 2249-1 (5a).

Growth of *E. cloacae* in mucosal medium: susceptibility to gentamicin. The ability of the test organisms to survive or multiply in the mucosal medium and the susceptibility of the organisms to gentamicin (100 μg/ml) under the conditions of the experiment were determined as described before (1).

Rabbit ileal invasion assay. This rabbit ileal invasion assay (apparatus, media, and solutions, etc.), originally developed for studying the invasiveness of *S. typhimurium*, has been described in detail (1). A brief description only of those details essential to the present study and minor changes to the standard protocol are described here. The design of the apparatus was as described previously (1) except that a 12-chamber model, instead of the original 8-chamber model, was used.

Preparation of the inocula. Organisms were retrieved as required from glycerol stock cultures held at −70°C and plated on MacConkey agar (Oxoid) plates and incubated overnight. A single colony was transferred from MacConkey agar to 10 ml of Hartley digest broth (Oxoid) and incubated statically at 37°C overnight. Bacteria were harvested by centrifugation (3,500 × *g*) and resuspended in 10 ml of fresh medium, 1 ml of which was transferred into 90 ml of Hartley digest broth and incubated statically at 37°C for 3 h. At this stage, for purely logistical reasons arising from the increase from 8 (1) to 12 chambers in the apparatus (and, as a consequence, the increased numbers of samples generated in the invasion assay to be handled in one working day), an additional step was introduced. Early-log-phase organisms (1, 6, 12) were centrifuged, washed, resuspended in 10 ml of fresh Hartley digest broth, counted, and stored on ice overnight. Immediately before the invasion assay was started, the organisms were centrifuged, resuspended in mucosal medium, mixed thoroughly to minimize clump formation, and adjusted to ca. 10^8 cells per ml. Viable counts on the inoculum were performed once more.

Organ culture system. The organ culture system consists of two Perspex halves (1), each containing 12 chambers. Between these halves, pieces of rabbit distal ileal mucosal epithelium were mounted all in the same orientation. The tissue for each experiment was freshly isolated from a New Zealand White rabbit (2.5 to 3.0 kg) and stripped of serosal muscle layers, avoiding Peyer's patches. Approximately 50 mm^2 of tissue was in contact with the bathing solutions in each chamber. Serosal chambers were filled with 3 ml of serosal medium, and mucosal chambers were filled with 3 ml of mucosal medium containing ca. 10^8 bacteria. Each chamber was gassed with a steady flow of O_2 (95%)–CO_2 (5%) during 2 h of incubation at 37°C. The bacterial suspension was replaced by fresh mucosal medium containing gentamicin (100 μg/ml), and incubation was continued for 1 h. Ileal tissue was removed from the apparatus, washed twice with saline, and homogenized on ice in 3 ml of 1% Triton X-100 (BDH) in phosphate-buffered saline (PBS) with a Sorvall Omni Mixer (for 30 s at maximum speed). Tenfold serial dilutions were made from the homogenized tissues, and viable counts on MacConkey agar were carried out.

Media and buffers. The serosal surface was bathed in the World Health Organization rehydration formulation: NaCl, 60 mM; $NaHCO_3$, 30 mM; KCl, 20 mM; and glucose, 111 mM. The mucosal surface was bathed in GC/Tcm medium, which is the same solution as the rehydration formula but with two important changes: all of the sodium was replaced by choline (GC medium) and the solution was supplemented with tissue culture medium (Tcm). Tcm is commercial minimum essential medium (Life Technologies) to which fetal calf serum and glutamine were added to final concentrations of 10% (vol/vol) and 2.0 mM, respectively. Tcm was added to the GC medium to a final concentration of 10% (vol/vol). This final product is called GC/Tcm medium. The rationale for the composition of these solutions has been fully explained (1).

PBS consists of 8 g of NaCl, 0.2 g of KCl, 2.16 g of $Na_2HPO_4 \cdot 7H_2O$, 0.24 g of KH_2PO_4 in 800 ml of distilled water (with the pH adjusted to 7.4 with H_3PO_4), and distilled water added to 1 liter.

Invasiveness. Invasiveness was expressed as that percentage of the original inoculum added to the chamber which was recovered from each piece of tissue. A known invasive organism (*S. typhimurium* TML) and a known noninvasive organism (*S. typhimurium* LT7) were included in some assays to validate the technique in the context of these experiments with *E. cloacae* and to assess the relative invasiveness of *E. cloacae*, an opportunistic pathogen, against that of an invasive pathogen. In the other assays, the comparisons were made between *E. cloacae* strains as a function of their OmpX contents. Because of uncontrollable variability in the susceptibility to invasion of gut tissue in different animals, a direct comparison of data for different strains can be obtained only with gut from the same rabbit. While it is true that absolute invasiveness varies, the pattern of relative invasiveness exhibited between strains does not. Therefore, interexperimental comparisons are made by including appropriate internal standards and normalizing the results to this standard (1).

RESULTS

Growth of *E. cloacae* in mucosal medium: susceptibility to gentamicin. The validity of the assay depends on the ability of the test organisms to survive or multiply in the mucosal medium and on their susceptibility to gentamicin under the conditions of the experiment. The survivability and growth of *E. cloacae* strains (Fig. 1) were comparable to that of *S. typhimurium* TML and *S. typhimurium* LT7 shown in earlier work (1).

E. cloacae was demonstrably susceptible to gentamicin (100 μg/ml in mucosal medium): after 1 h of incubation at 37°C, the viable count dropped from 10^7 to $<10^2$ ml^{-1}. On the basis of the data for *S. typhimurium* (1) in organ culture, one can be fairly confident that all extracellular *E. cloacae* organisms in

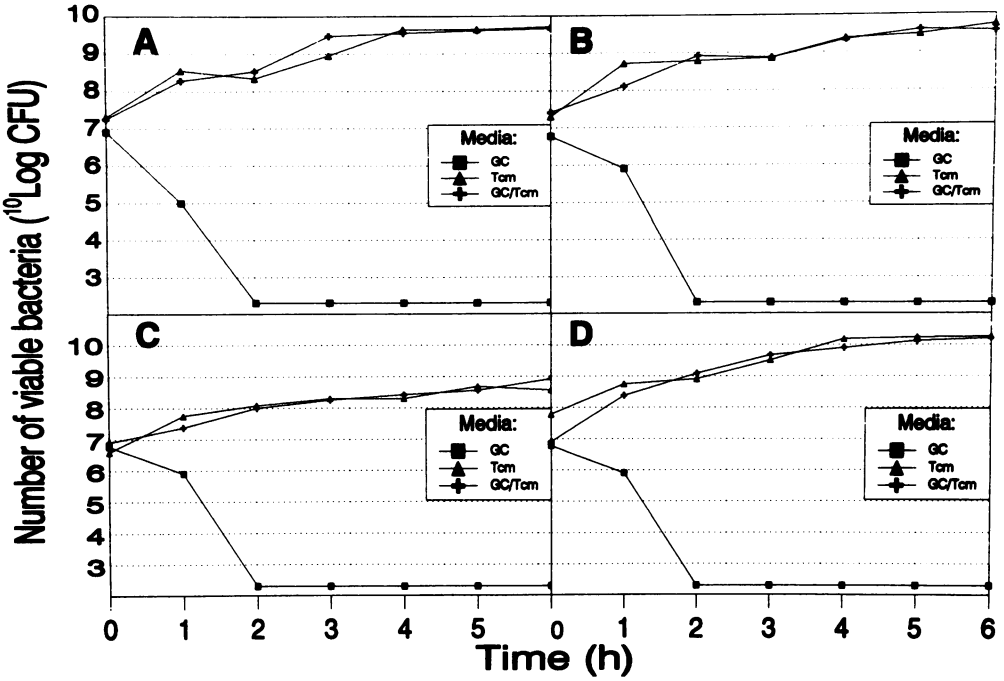

FIG. 1. Viability of organisms in the test media. (A) *E. cloacae* JS101; (B) *E. cloacae* JS101(pACYC184); (C) *E. cloacae* JS101(pJS04); (D) *E. cloacae* 2249-1. The compositions of the media GC, Tcm, and GC/Tcm are described in Materials and Methods.

the organ culture system would be killed and, hence, that organisms resistant to gentamicin are intracellular.

Relation of inoculum to invasiveness. For this method of assessing invasiveness to be valid, it is essential that a linear relationship exists between inoculum size and the numbers of organisms which become internalized: this was demonstrable with *E. cloacae* JS101 over a wide inoculum range, 8.0×10^6, 6.0×10^7, and 2.5×10^8 CFU, respectively (Fig. 2). This type of relationship has also been firmly established with *S. typhimurium*, *Salmonella dublin*, and *Salmonella choleraesuis* over a greater range, i.e., up to 1.5×10^9 CFU (4a); on this basis, we feel justified in extrapolating the *E. cloacae* data as was necessary in experiments with rabbits F and G (see below and Fig. 4).

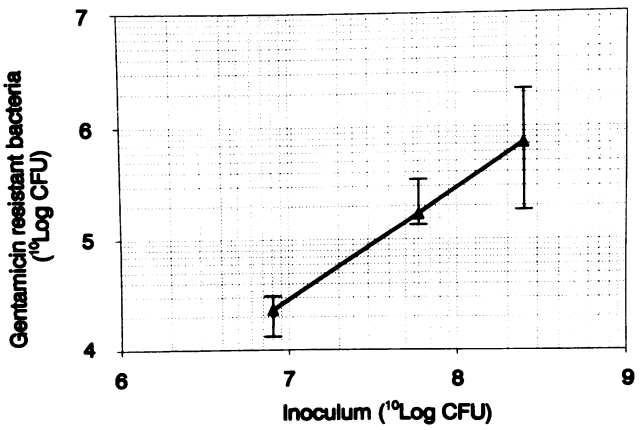

FIG. 2. Correlation of inoculum size with recovery of organisms from rabbit gut. Organisms were added and left for 2 h, after which supernatants were removed from the chambers and the gut tissue was incubated for 1 h with gentamicin. Strain *E. cloacae* JS101 was used in this experiment. The regression line has a correlation coefficient of 0.90. Symbol: ▲, marker of the regression line.

Invasiveness of wild-type *E. cloacae* (strain 2249-1) and the *ompX* deletion mutant (strain JS101). Experiments were carried out with *E. cloacae* 2249-1 and JS101 and the *Salmonella* control strains (TML and LT7) with ileal tissue prepared from four rabbits, A, B, C, and D, with each rabbit being used in separate experiments on separate days (Fig. 3, top). Not surprisingly, the absolute levels of invasiveness of strains varied. Nevertheless, a consistent pattern of relative invasiveness emerged (Fig. 3, bottom). There was no statistically significant difference between the two *E. cloacae* strains (2249-1 and JS101). The invasiveness of the *E. cloacae* strains was comparable to (Fig. 3B and C) or 5 to 10 times less than (Fig. 3A and D) that of the *S. typhimurium* strain LT7.

Invasiveness of the OmpX overproducer and the *ompX* deletion mutant. In a second set of experiments, the invasivenesses of the *ompX* deletion mutant (JS101), the vector-only strain [JS101(pACYC184)], and the OmpX-overproducing strain [JS101(pJS04)] of *E. cloacae* were compared; *S. typhimurium* TML and LT7 or TML by itself was carried as the control. Ileal tissue was prepared from four rabbits, namely, E, F, G, and H, with each rabbit being used in separate experiments on separate days. In rabbits E and F, the OmpX-overproducing strain was compared with the OmpX-minus strain (Fig. 4). In rabbits G and H, to exclude the influence of the vector moiety of the pJS04 construct, the overproducing strain was compared with *E. cloacae* JS101(pACYC184) (Fig. 4). In these experiments, the invasiveness of the OmpX overproducer was at least 10-fold higher than that of *E. cloacae* JS101(pACYC184) in rabbits E, F, and H and at least 1.5 times higher in rabbit G ($P = 0.015$; see Fig. 4 legend for comment). In tissues from rabbits E and F, the invasiveness of the OmpX overproducer was in both cases 4.5 times higher than that of *S.*

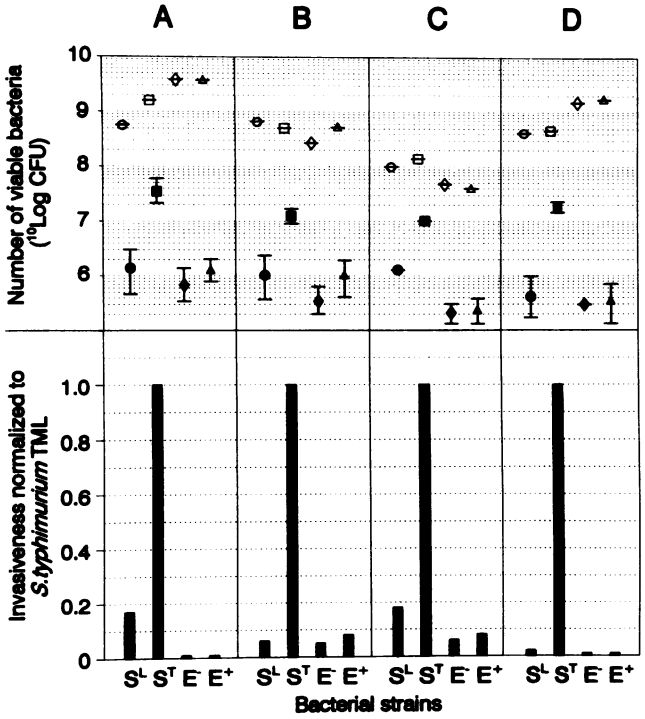

FIG. 3. Comparison of invasivenesses of the *Salmonella* strains, wild-type *E. cloacae*, and the OmpX deletion mutant. (A, B, C, and D) Results from rabbits A, B, C, and D, respectively. (Top) Inoculum and mean recovery for each strain in the different rabbits; (bottom) data presented as invasiveness (see Materials and Methods) and normalized to the results with the invasive organism *S. typhimurium* TML. The bottom panels directly display the differences in invasiveness between *S. typhimurium* TML and the other strains. Abbreviations and symbols: ○ and ●, *S. typhimurium* LT7 (S^L); □ and ■, *S. typhimurium* TML (S^T); ◇ and ◆, *E. cloacae* JS101 (E^-); △ and ▲, *E. cloacae* 2249-1 (E^+); open symbols, inoculum; filled symbols, organisms recovered from gut mucosa. Vertical bars indicate the standard errors of the means from $n = 6$ measurements per strain, with the following exceptions: panel B, TML ($n = 3$), JS101 ($n = 10$), 2249-1 ($n = 10$), panel C; TML ($n = 5$).

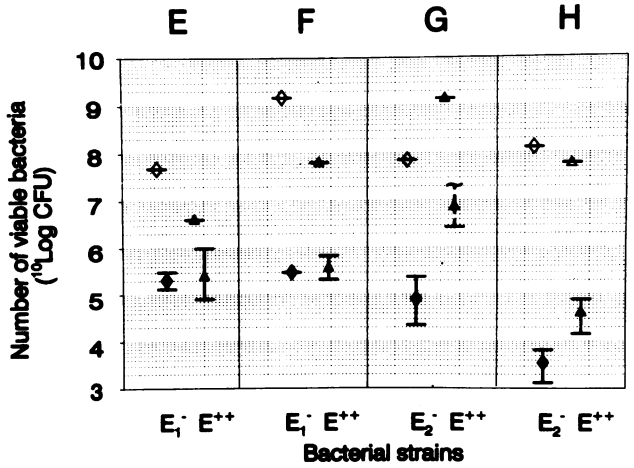

FIG. 4. Comparison of invasivenesses of the OmpX-overproducing *Enterobacter* strain and OmpX deletion mutants. (E, F, G, and H) Results from rabbits E, F, G, and H, respectively. Abbreviations and symbols for panels E and F: ◇ and ◆, *E. cloacae* JS101 (E_1^-); △ and ▲, *E. cloacae* JS101(pJS04) (E^{++}). Abbreviations and symbols for panels G and H: ◇ and ◆; *E. cloacae* JS101(pACYC184) (E_2^-); △ and ▲, *E. cloacae* JS101(pJS04) (E^{++}). Symbols for all panels: open symbols, inoculum; filled symbols, organisms recovered from gut mucosa. Vertical bars indicate standard errors of the means from $n = 6$ measurements per strain with the following exceptions: panel E, JS101(pJS04) ($n = 12$); panel F, JS101(pJS04) ($n = 12$); panel G, JS101(pJS04) ($n = 12$) and JS101(pACYC184) ($n = 12$). The recovery of the OmpX-overproducing strain (E^{++}) in rabbit G was at least 2.7×10^6 CFU. In this experiment, a larger than usual inoculum gave rise to increased numbers of gentamicin-resistant organisms. The dilution series was misjudged. The value recorded represents a minimum estimate of recovered organisms. The true invasiveness was therefore greater than was estimated. This is reflected in this figure with a broken vertical line (G) and in Fig. 5 with a wavy horizontal line (G).

typhimurium LT7 but lower than that of *S. typhimurium* TML (1.2 and 8.8 times, respectively); the invasiveness of *E. cloacae* JS101(pACYC184) was three and five times lower than that of *S. typhimurium* LT7 in rabbits E and F, respectively. In the experiments with tissues from rabbits G and H, the *Enterobacter* strains were also less invasive than *S. typhimurium* TML.

Data for wild-type and OmpX-overproducing *E. cloacae* strains, expressed as invasiveness and normalized to data for *E. cloacae* JS101 (*ompX* deletion mutant), are shown in Fig. 5.

DISCUSSION

This is only the second bacterial system to be studied in this new assay for invasion of gut enterocytes in situ by an enteric organism. In every case where *S. typhimurium* TML and LT7 were used, the same relative invasiveness was obtained as that reported earlier (1). Several points emerge from this work in relation to *E. cloacae*.

First, there is little difference between the invasiveness of the wild-type strain and the *ompX* deletion mutant when grown under laboratory conditions. Moreover, comparison with *S. typhimurium* LT7 demonstrates that *E. cloacae* is normally poorly invasive at least under these test conditions. This might be expected since *E. cloacae* belongs to the commensal flora of the human gut, which implies an ability to colonize but not to invade gut mucosa.

Second, increased expression of *ompX* in the overproducing mutant correlated positively with an increased ability to invade normal gut, which was greater than that of *S. typhimurium* LT7 but less than that of *S. typhimurium* TML. In view of the arguments set forth in the introduction predicting the biological properties of OmpX, it is not unreasonable to assign a causal relationship between high *ompX* expression and invasiveness.

It is now possible to interpret, albeit in a speculative manner, the pathogenic attributes of *E. cloacae* 2249-1 used in this study in terms of an upregulated expression of *ompX*. This strain was a clinical isolate from a patient receiving cefamandole therapy (21). Small amounts of cefamandole are known to be excreted with bile into the intestinal lumen (17). This obviously would create selection pressure for the emergence of the observed antibiotic resistance. Since the observed antibiotic resistance is now explicable in terms of *ompX* expression, it would not be unreasonable to postulate that the antibiotic itself upregulates the expression of *ompX* at least under the conditions prevailing in the gut. If this were to happen, and since we have now demonstrated that an organism manipulated to overexpress *ompX* under laboratory conditions will exhibit increased invasiveness towards normal gut, one would expect an increase in the invasive potential of such a phenotype. However, the

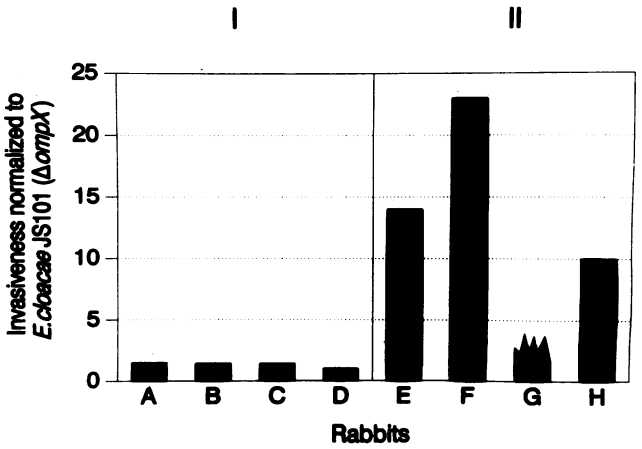

FIG. 5. Invasivenesses of *E. cloacae* strains with different levels of OmpX, normalized to *ompX* deletion strains. (I) Wild-type *E. cloacae* 2249-1 normalized to *E. cloacae* JS101 (see the legend to Fig. 3); (II) OmpX-overproducing *E. cloacae* JS101(pJS04) normalized to the *ompX* deletion mutant *E. cloacae* JS101 in rabbits E and F and to *E. cloacae* JS101(pACYC184) in rabbits G and H. The normalized invasiveness of *E. cloacae* JS101(pJS04) in rabbit G is at least 1.5 (see legend to Fig. 4 for comment).

observed increased invasiveness was not comparable to that of *S. typhimurium* TML, although it was greater than that of strain LT7. However, the patient from which the isolate was obtained was receiving antibiotic therapy while being treated with chemotherapeutic agents. Since anticancer agents have a predilection for rapidly dividing cells, this would affect the gut, as has been already demonstrated in previous experimental work with rabbits and *S. typhimurium* invasion: the numbers of organisms invading such gut epithelia increased enormously (24).

The nature of the invasion assay is such that one measures the numbers of surviving intracellular organisms at least over the time of the assay: it is clear that *E. cloacae* organisms survive. Thus, organisms could escape and become systemic where OmpX would provide protection from the effects of complement-mediated killing and further attack by the antibiotic, an ingenious survival strategy.

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