

Synthesis of Metallo- β -Lactamase VIM-2 Is Associated with a Fitness Reduction in *Salmonella enterica* Serovar Typhimurium

Nicolás F. Cordeiro,^a José A. Chabalgoity,^b Lucía Yim,^b  Rafael Vignoli^a

Departamento de Bacteriología y Virología, Instituto de Higiene, Facultad de Medicina, Universidad de la República, Montevideo, Uruguay^a; Departamento de Desarrollo Biotecnológico, Instituto de Higiene, Facultad de Medicina, Universidad de la República, Montevideo, Uruguay^b

Antibiotic resistance, especially due to β -lactamases, has become one of the main obstacles in the correct treatment of *Salmonella* infections; furthermore, antibiotic resistance determines a gain of function that may encompass a biological cost, or fitness reduction, to the resistant bacteria. The aim of this work was to determine *in vitro* if the production of the class B β -lactamase VIM-2 determined a fitness cost for *Salmonella enterica* serovar Typhimurium. To that end the gene *bla*_{VIM-2} was cloned into the virulent strain *S. Typhimurium* SL1344, using both the tightly regulated pBAD22 vector and the natural plasmid pST12, for inducible and constitutive expression, respectively. Fitness studies were performed by means of motility, growth rate, invasiveness in epithelial cells, and plasmid stability. The expression of *bla*_{VIM-2} was accompanied by alterations in micro- and macroscopic morphology and reduced growth rate and motility, as well as diminished invasiveness in epithelial cells. These results suggest that VIM-2 production entails a substantial fitness cost for *S. Typhimurium*, which in turn may account for the extremely low number of reports of metallo- β -lactamase-producing *Salmonella* spp.

The genus *Salmonella* includes pathogenic serovars that produce a variety of diseases in humans and animals (1). Antibiotic treatment of nontyphoidal salmonellosis is usually indicated for severe infections in children, elderly people, and immunocompromised patients; therapeutic options include folate inhibitors, fluoroquinolones, and oxyiminocephalosporins (2). The development of strains with increasing levels of resistance due to widespread use of antibiotics has been one of the main setbacks for the effective treatment of *Salmonella* infections (3). Most of the clinical isolates of β -lactamase-producing *Salmonella* synthesize class A β -lactamases (4), and the occurrence of isolates carrying plasmidic class C β -lactamases has also been reported worldwide (5, 6). Regarding class D β -lactamases, only a few variants have been reported in *Salmonella* serotypes (e.g., OXA-1, -2, -53, -129, and -48) (7, 8, 9). In addition, the occurrence of metallo- β -lactamase (MBL)-producing salmonellae is an infrequent event. In recent years, a few clinical cases have been reported, all of them corresponding to NDM-producing *Salmonella* (10, 11, 12). Interestingly, VIM alleles were not reported in *Salmonella* isolates until 2013 (9, 13).

Other authors have studied the biological cost for *Salmonella enterica* serovar Typhimurium associated with the production of class A, C, and D β -lactamases (14); nevertheless, there is no information regarding the effects of the synthesis of MBL in such microorganisms.

In this work, we hypothesized that the production of VIM-2 entailed a biological cost for *Salmonella*. To address this question, we analyzed the effects caused by VIM-2 production in *S. Typhimurium* on parameters such as motility, growth rate, and eukaryotic cell invasiveness. The class D carbapenemase OXA-66 was used as a control since it showed no deleterious effects on the fitness of *S. Typhimurium*.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. *S. Typhimurium* SL5338 (15), a strain deficient in all three restriction systems, was the mediator to transform plasmid DNA into strain *S. Typhimurium* SL1344

(16), used for all the *in vitro* experiments. The *Escherichia coli* strains used were DH5 α (17) and MC1061 (18) and clinical isolate NF7 (19). Genes *bla*_{OXA-66} and *bla*_{VIM-2} were obtained from *Acinetobacter baumannii* strain 39AT (20) and *Pseudomonas aeruginosa* strain I42, respectively (21); the latter strain was kindly provided by Juan Ayala (Centro de Biología Molecular Severo Ochoa, Universidad Autónoma de Madrid, Spain). A detailed description of the bacterial strains used in this study is given in Table 1.

All strains were grown aerobically at 37°C in Luria-Bertani (LB) broth or agar (Amresco, Solon, OH) supplemented with 100 μ g/ml ampicillin (AMP) or 25 μ g/ml kanamycin (KAN) (Sigma-Aldrich, St. Louis, MO) when required.

DNA manipulation. Genetic manipulation was performed using standard laboratory techniques. Plasmids and chromosomal DNA were purified following the manufacturer's recommendations (QIAprep Spin miniprep kit and DNeasy blood and tissue kit; Qiagen Sample Assay Technologies). DNA restriction and ligation assays were carried out under standard conditions per the manufacturer's instruction (Fermentas, Invitrogen). Preparation of competent *E. coli* and electrocompetent *S. Typhimurium* cells and DNA transformation were performed as previously described (22).

In order to assess the effects of VIM-2 production on bacterial fitness, we cloned the *bla*_{VIM-2} gene into pBAD22 under the control of the arabinose-regulated P_{BAD} promoter (23); additionally, *bla*_{OXA-66} was cloned into pBAD22 as a control, thus obtaining plasmids pLV1 and pLO5 (Table 1). Construction of pLO5 and pLV1 was performed as follows. Genes *bla*_{OXA-66} and *bla*_{VIM-2} were amplified using the primers *oxaF* and *oxaR*

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Address correspondence to Rafael Vignoli, rvignoli@higiene.edu.uy.

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TABLE 1 Bacterial strains and plasmids used in this study

| Strain or plasmid | Description ^a | Reference or source |
|--|---|---------------------|
| <i>S. Typhimurium</i> strains | | |
| SL1344 | <i>xyl hisG rpsL</i> Sm ^r | 16 |
| SL5338 | <i>galE</i> r ⁻ m ⁺ | 15 |
| <i>E. coli</i> strains | | |
| DH5 α | F ⁻ <i>endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG ϕ80dlacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>)U169 <i>hsdR17</i> (r _K ⁻ m _K ⁺) λ ⁻ | 17 |
| MC1061 | F ⁻ Δ (<i>ara-leu</i>)7697 [<i>araD139</i>] _{B/r} Δ (<i>codB-lacI</i>)3 <i>galK16 galE15</i> λ ⁻ <i>e14⁻ mcrA0 relA1 rpsL150 (strR) spoT1 mcrB1 hsdR2</i> (r ⁻ m ⁺) | 18 |
| NF7 | Uropathogenic <i>E. coli</i> clinical isolate, receptor strain | 19 |
| <i>P. aeruginosa</i> strain I42 | Template strain, <i>bla</i> _{VIM-2} | 21 |
| <i>Acinetobacter calcoaceticus</i> - <i>A. baumannii</i> complex strain 39AT | Template strain, <i>bla</i> _{OXA-66} | 20 |
| Plasmids | | |
| pBAD22 | Expression vector with P _{BAD} and AraC control (Ap ^r) | 23 |
| pLV1 | <i>bla</i> _{VIM-2} inserted between sites EcoRI and HindIII of pBAD22 | This work |
| pLO5 | <i>bla</i> _{OXA-66} inserted between sites EcoRI and XbaI of pBAD22 | This work |
| pST12 | Environmental plasmid with <i>bla</i> _{TEM-144} under the control of P ₃ promoter | 25 |
| pSTVIM | <i>bla</i> _{VIM-2} inserted between sites PstI of plasmid pST12 | This work |
| pKD4 | Template plasmid, pANTS γ derivative (Ap ^r Kn ^r) | 24 |
| pBAD22 <i>bla</i> _{TEM-1::kan} | <i>aph</i> (3') inserted in site PvuI of pBAD22 (Kn ^r) | This work |
| pLO5 <i>bla</i> _{TEM-1::kan} | <i>aph</i> (3') inserted in site PvuI of pLO5 | This work |
| pLV1 <i>bla</i> _{TEM-1::kan} | <i>aph</i> (3') inserted in site PvuI of pLV1 | This work |

^a Sm^r, streptomycin resistant; Ap^r, ampicillin resistant; Kn^r, kanamycin resistant.

and *vimF* and *vimR* (see Table S1 in the supplemental material), using whole genomic DNA from *A. baumannii* strain 39AT and *P. aeruginosa* I42, respectively, as the template. PCR products of *bla*_{OXA-66} and *bla*_{VIM-2} were then doubly digested with EcoRI/XbaI and EcoRI/HindIII (Fermentas Life Sciences, Vilnius, Lithuania), respectively, and cloned into plasmid pBAD22. Insert identity was assessed by sequencing with primers pBAD5 and pBADrev (see Table S1 in the supplemental material).

Later, the *bla*_{TEM} gene endogenous to pBAD22 was inactivated to avoid possible interferences between the β -lactamases in the resulting constructs, thus yielding pBAD22 *bla*_{TEM-1::kan}, pLO5 *bla*_{TEM-1::kan}, and pLV1 *bla*_{TEM-1::kan}. These plasmids were constructed by inserting the kanamycin resistance gene *aph*(3') into a PvuI recognition site in the *bla*_{TEM-1} gene present in pBAD22, pLO5, and pLV1, respectively (Table 1). Plasmid pKD4 (24) served as a template for PCR amplification of *aph*(3') using primers P1pvu and P2pvu (see Table S1 in the supplemental material).

Additionally, constitutive *bla*_{VIM-2} expression was achieved by cloning said gene into a natural plasmid. Plasmid pST12 (accession number HG428760) is a ColE1-like plasmid obtained from an environmental *Salmonella enterica* serovar Derby isolate, featuring *bla*_{TEM-144} under the regulation of promoter P₃ and three recognition sites for the restriction enzyme PstI (25). Plasmid pSTVIM is a derivative of pST12 carrying *bla*_{VIM-2} in place of *bla*_{TEM-144}. Briefly, plasmid pST12 was digested with PstI, generating three fragments of 4.76 kb (containing the backbone of pST12), 2.85 kb (containing the extended-spectrum β -lactamase [ESBL] gene *tnpR* and part of *tnpA*), and 0.66 kb (containing another fragment of *tnpA*); the 4.76-kb fragment was recovered by gel excision and treated with alkaline phosphatase to prevent religation. Then a P₃ promoter-*bla*_{VIM-2} fusion was created; P₃ and *bla*_{VIM-2} were amplified with primers prmF and pvmRV and vmpFW and vmpRV, respectively (see Table S1 in the supplemental material). Primer pvmRV has a 3' 20-mer region exhibiting perfect homology with the 5' region of primer vmpFW. Next, an equimolar mixture of both PCR products was used as the template for

PCR fusion of P₃-*bla*_{VIM-2}; after 2 cycles of amplification aimed at the hybridization of the promoter with the MBL-coding gene, primers prmF and vmpRV were added to the reaction mix, allowing the PCR to proceed for another 30 cycles. The P₃-*bla*_{VIM-2} hybrid was treated with PstI and then ligated to the 4.7-kb fragment of pST12. Sealing of 5' nicks was achieved by transforming pSTVIM into *E. coli* MC1061 prior to its final electroporation into *S. Typhimurium* SL1344. Insert identity was confirmed by sequencing using primers pSTF and pSTr (see Table S1 in the supplemental material).

Finally, for comparison purposes, plasmids pBAD22 *bla*_{TEM-1::kan}, pLO5 *bla*_{TEM-1::kan}, pLV1 *bla*_{TEM-1::kan}, pST12, and pSTVIM, were also electroporated into *E. coli* strain NF7.

Analysis of β -lactamase production and determination of β -lactamase activity. Crude extracts were obtained from strains SL1344(pBAD22 *bla*_{TEM-1::kan}), SL1344(pLO5 *bla*_{TEM-1::kan}), and SL1344(pLV1 *bla*_{TEM-1::kan}), both in the presence and in the absence of 0.2% (wt/vol) L-arabinose, to analyze enzyme production and to assess β -lactamase activity of the different constructs. Briefly, overnight (ON) cultures in LB broth with kanamycin were diluted 1/100-fold in fresh preheated LB with kanamycin and incubated for another 90 min; then L-arabinose was added and induced cultures were further incubated for 2 h at 37°C with shaking (200 rpm). Strain SL1344(pBAD22) was cultivated in parallel in the presence of ampicillin. Cells were then harvested at 4,000 rpm, washed with sterile phosphate-buffered saline (PBS), and resuspended in buffer (Tris 10 mM and NaCl 50 mM [pH 7.5]), and sonicated at 0°C. Finally, the extracts were centrifuged for 30 min at 10,000 rpm at 4°C, and the supernatant was recovered.

Protein concentration was estimated by Bradford's method (26), following the manufacturer's instructions (Sigma, Saint Louis, MO), and ~50 μ g of proteins of each crude extract was analyzed by 15% SDS-PAGE with Coomassie stain. Accordingly, the crude extract of induced strain SL1344(pLV1 *bla*_{TEM-1::kan}) showed an increase in intensity of an ~28-

TABLE 2 MICs to ceftazidime and imipenem for inducible and constitutive VIM-2 producing strains

| Strain | MIC (mg/liter) | |
|--|-----------------|--------------------------------|
| | Uninduced | Induced (0.05% L-arabinose) |
| SL1344(pBAD22 <i>bla</i> _{TEM-1} ::kan) | | |
| Ceftazidime | 0.25 | 0.25 |
| Imipenem | 0.023 | 0.023 |
| SL1344(pLV1 <i>bla</i> _{TEM-1} ::kan) | | |
| Ceftazidime | 0.5 | 1 |
| Imipenem | 0.032 | 0.75 |
| SL1344(pLV1 <i>bla</i> _{TEM-1} ::kan [mRNA (2 ^{ΔCT})]) | 0.15 | 78.61 |
| SL1344(pST12) | 64 ^a | 0.035 ^b |
| SL1344(pSTVIM) | 12 ^a | 0.125 ^b |

^a MIC of ceftazidime.^b MIC of imipenem.

kDa band, compatible with VIM-2 (predicted molecular weight, 28.3 kDa) and suggestive of the induced synthesis of such enzymes.

β-Lactamase activity was then assessed by the iodometric overlay method (27) using 500 μg/ml ampicillin as the substrate; while the crude extract of the induced strain SL1344(pLV1 *bla*_{TEM-1}::kan) showed immediate β-lactamase activity (~30 s), no activity was detected with the extract of the uninduced strain SL1344(pLV1 *bla*_{TEM-1}::kan), indicating arabinose-regulated VIM-2 production. On the other hand, the crude extracts of control strains SL1344(pBAD22) and induced SL1344(pLO5 *bla*_{TEM-1}::kan) also displayed β-lactamase activity, albeit not as readily as SL1344(pLV1 *bla*_{TEM-1}::kan). The crude extract of strain SL1344(pBAD22 *bla*_{TEM-1}::kan) showed no activity, with or without induction.

Antibiotic susceptibility testing. Antibiotic susceptibility profiles of strains harboring the various constructs were determined by disk diffusion assay following the Clinical and Laboratory Standards Institute (CLSI) guidelines (28). MIC values for ceftazidime and imipenem were determined by Etest (bioMérieux, Marcy l'Etoile, France), following the manufacturer's instructions. Results were interpreted according to CLSI breakpoints (28). When necessary, Mueller-Hinton plates for antibiograms and MIC assays were supplemented with 0.05% (wt/vol) L-arabinose. Additionally, EDTA disks (2.5 μM) were also used to assess the inhibitory effects on the MBL VIM-2. *S. Typhimurium* strains SL1344 wild type (WT), SL1344(pBAD22), SL1344(pBAD22 *bla*_{TEM-1}::kan), and SL1344(pST12) were included as controls. As expected, the VIM-2-producing strains SL1344(pLV1 *bla*_{TEM-1}::kan) (inducible) and SL1344(pSTVIM) (constitutive) showed activity against oxyiminocephalosporins and carbapenems; additionally, a characteristic synergy effect between the EDTA disk and β-lactam-containing disks was observed. On the other hand, strain SL1344(pBAD *bla*_{TEM-1}::kan) showed resistance only to kanamycin, whereas strain SL1344(pLO5 *bla*_{TEM-1}::kan) showed resistance only to ampicillin and cephalothin (data not shown). MIC results are displayed in Table 2.

Detection of alterations in colony morphology. Ten microliters of bacterial suspensions of strains SL1344(pBAD22), SL1344(pLO5), and SL1344(pLV1) containing 1.5×10^4 CFU/ml (~150 CFU) was streaked onto 5% sheep blood agar containing increasing concentrations of L-arabinose (0%, 0.05%, and 0.1%) and supplemented with 100 μg/ml ampicillin. Plates were incubated aerobically ON at 37°C. Similarly, strains SL1344(pST12) and SL1344(pSTVIM) were streaked onto 5% sheep blood agar plates without L-arabinose.

Motility tests. Swimming motility assays were performed for strains SL1344(pBAD22 *bla*_{TEM-1}::kan), SL1344(pLO5 *bla*_{TEM-1}::kan), and

SL1344(pLV1 *bla*_{TEM-1}::kan), according to Cordeiro et al. (6), in LB plates containing 0.3% agar and increasing concentrations of L-arabinose (0%, 0.05% and 0.1%) and were incubated for 6 h at 37°C. Motility assays for strains SL1344(pST12) and SL1344(pSTVIM) were performed similarly, albeit with plating onto 0.3% LB agar without L-arabinose. The diameter of the motility halo was measured after 6 h of incubation. Assays were performed in triplicate.

Growth curves. Growth curves for β-lactamase-producing strains were performed as previously described (6) for strains SL1344(pBAD22 *bla*_{TEM-1}::kan), SL1344(pLO5 *bla*_{TEM-1}::kan), and SL1344(pLV1 *bla*_{TEM-1}::kan) in LB supplemented with kanamycin and with and without 0.05% (wt/vol) L-arabinose. Growth curves for strains SL1344(pST12) and SL1344(pSTVIM) were performed with LB broth supplemented with ampicillin, and no L-arabinose was added.

Additionally, growth curves for strains NF7(pBAD22 *bla*_{TEM-1}::kan) and NF7(pLV1 *bla*_{TEM-1}::kan) were performed in kanamycin-supplemented LB with and without arabinose, whereas those for strains NF7(pST12) and NF7(pSTVIM) were performed in ampicillin-supplemented LB broth.

In all cases, measurements of the optical density at 600 nm (OD₆₀₀) were taken at regular intervals (30 min) until the stationary growth phase. Cultures were diluted every 60 min with fresh preheated LB (supplemented with kanamycin or ampicillin and arabinose) to maintain the cultures in the exponential phase for approximately 3 h. All assays were performed in triplicate.

Cell invasion assays. Bacterial viability in cell tissue culture medium, as well as susceptibility to gentamicin, was determined for every strain prior to the invasion assays in the Caco-2 cell line. Caco-2 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and were maintained in minimal essential medium with Earle's salts (high glucose, 4.5 g/liter) (PAA, Pasching, Austria) supplemented with 2 mM L-glutamine (Sigma-Aldrich) and 15% fetal bovine serum (PAA, Pasching, Austria). Cells were grown at 37°C in 5% CO₂, at up to 80% confluence. Caco-2 invasion assays were performed as previously described (6). Strains SL1344(pBAD22), SL1344(pLO5), SL1344(pLV1), SL1344(pST12), and SL1344(pSTVIM) were grown accordingly until the log phase (OD₆₀₀ ~0.4), as described in the growth curves section. Each strain was tested in triplicate in three independent experiments.

Quantitative real-time PCR of *bla*_{OXA-66} and *bla*_{VIM-2}. Fresh cultures of strains SL1344(pBAD22), SL1344(pLO5), and SL1344(pLV1) in LB containing ampicillin were induced with 0.05% (wt/vol) L-arabinose during 60 min (up to an OD₆₀₀ of ~0.4 to 0.8), and total RNA extraction was performed using an RNeasy Protect bacterial kit (Qiagen) according to manufacturer recommendations. Cultures grown in the same conditions but without arabinose were grown in parallel. Strains SL1344(pSTVIM) and *S. Typhimurium* SL1344 WT were incubated similarly but without L-arabinose (the latter in the absence of ampicillin).

Next, 100 ng of the resulting RNA was treated with DNase (Invitrogen, Carlsbad, CA) following the manufacturer's instructions and further reverse transcribed using Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen) and random hexamer primers in a 20-μl reaction mixture. Then 2 μl of a 1/16 dilution of this mixture was used for quantitative real-time PCR (qRT-PCR) using SYBR green (QuantiTect; Qiagen) in a Corbett Rotor-Gene 6000 (Qiagen). Primer sequences are detailed in Table S1 in the supplemental material. The cycling program was 15 min at 95°C and 45 cycles of 15 s at 94°C, 30 s at 57°C, and 30 s at 72°C. The comparative threshold cycle (*C_T*) method was used for relative mRNA quantification (29) using the *bla*_{TEM-1} gene present on pBAD22 and derivatives as an endogenous reference to avoid errors derived from plasmid copy number variations. Briefly, for strains SL1344(pLV1) and SL1344(pLO5), the increase in mRNA for *bla*_{VIM-2} and *bla*_{OXA-66}, respectively, was calculated as the 2^{ΔΔCT} or fold change [2^{ΔΔCT} (induced)/2^{ΔCT} (uninduced)]. For strain SL1344(pSTVIM) (constitutive expression), the Δ*C_T* was calculated as Δ*C_T* = *C_T*_{VIM-2} - *C_T*_{VIM-1}, where *C_T*_{VIM-2}

is the threshold cycle obtained for the endogenous control (16S rRNA gene) and C_{TVIM} is the threshold cycle obtained for bla_{VIM-2} .

In this regard, we detected a 520-fold increase of bla_{VIM-2} mRNA for strain SL1344(pLV1) and a 310-fold increase of bla_{OXA-66} mRNA for strain SL1344(pLO5) when strains were grown in the presence of arabinose relative to the absence of induction. Constitutive production of VIM-2 [strain SL1344(pSTVIM)] was also measured by qRT-PCR. In the absence of an induced and uninduced condition (i.e., constitutive expression), we calculated the $2^{-\Delta CT}$ value instead of the more commonly used $2^{-\Delta\Delta CT}$ (fold change). cDNA from strains *S. Typhimurium* SL1344 WT and SL1344(pST12) were used as negative controls. In this regard, the $2^{-\Delta CT}$ value obtained for strain SL1344(pSTVIM) was $3.25E-4 \pm 1.33E-4$; this value was two orders of magnitude lower than that obtained for strain SL1344(pLV1) (data not shown). Such differences occurred because bla_{VIM-2} in the plasmid pSTVIM is under the control of a weak promoter (30).

Plasmid stability assay. Plasmid stability assays were performed following a modified version of the procedure described by Medina et al. (31). Briefly, ON cultures in LB broth supplemented with kanamycin were diluted 1/200-fold in preheated LB broth; dilutions were incubated for 60 min at 37°C with shaking (200 rpm). Each culture was then fractionated in two; one half was induced with 0.05% (wt/vol) L-arabinose, whereas the other was repressed with 0.05% (wt/vol) glucose. Both sets of cultures were further incubated for another 11 h. Cultures were then diluted 1/200-fold every 12 h two times in fresh preheated LB broth with the corresponding amounts of L-arabinose or glucose. Strains SL1344(pST12) and SL1344(pSTVIM) were grown similarly, albeit without arabinose or glucose. Finally, samples were collected at an OD_{600} of ~ 0.8 (23 generations estimated) and diluted in sterile PBS, and equal volumes (10 μ l) were plated in LB agar with and without kanamycin. The proportion of plasmid-carrying bacteria was calculated as the number of CFU obtained in LB agar supplemented with kanamycin versus the CFU obtained in LB agar without antibiotic. All assays were performed in triplicate.

Statistical analysis. Numerical results obtained during this study were analyzed with the aid of SPSS 17.0 software (SPSS, Inc., Chicago, IL). Differences in motility, growth curves, and invasiveness in Caco-2 cells were compared by one-way analysis of variance (ANOVA). The Bonferroni adjustment was used as a *post hoc* test. Homoscedasticity was assessed by Levene's test of homogeneity of variances, and the Welch test was used whenever data showed heteroscedasticity. For all tests, differences were considered statistically significant for *P* values of < 0.05 (two-tailed).

RESULTS

Effects of VIM-2 production on macroscopic and cell morphology. In the presence of 0.05% L-arabinose, strain SL1344(pLV1) produced transparent and flattened colonies with irregular edges (Fig. 1a, right). Likewise, wet-mount microscopy of log-phase cultures showed that cells producing VIM-2 appeared lysed and larger than the control strain and the uninduced strain, suggesting a defect in septation and/or segregation as well as an increased susceptibility to osmotic lysis (Fig. 1b, right). Constitutive production of VIM-2 [strain SL1344(pSTVIM)] was accompanied by alterations of cellular and colony morphology similar to those described for the induced strain SL1344(pLV1) (data not shown). Conversely, strains SL1344(pBAD22) and SL1344(pLO5) did not show any micro- or macroscopic alterations when grown in the presence or absence of L-arabinose (data not shown).

Effect of VIM-2 production on *Salmonella* motility. Strain SL1344(pLV1 $bla_{TEM-1}::kan$) displayed a statistically significant reduction in motility when grown in inducing versus noninducing conditions (0% L-arabinose, 44.5 ± 4.5 mm; 0.05% L-arabinose, 26.3 ± 4.1 mm), suggesting that the production of VIM-2 has a negative effect on the motility of *S. Typhimurium*. In opposition, strains SL1344(pBAD22 $bla_{TEM-1}::kan$) and SL1344(pLO5

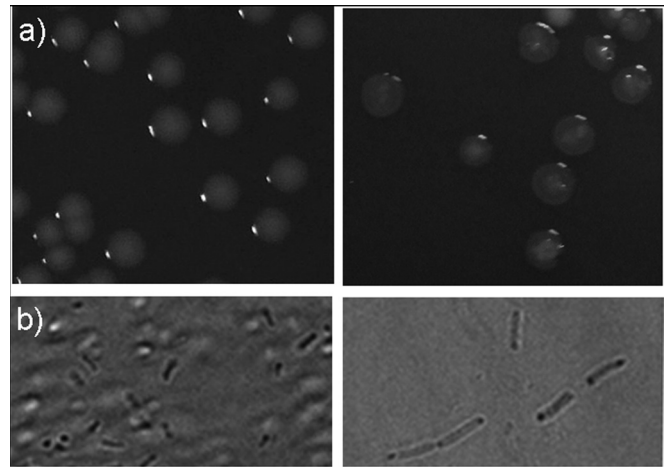


FIG 1 (a) Macroscopic morphology of *S. Typhimurium* SL1344 WT (left) and VIM-2-producing strain SL1344(pLV1) in the presence of arabinose (right). (b) Wet-mount microscopy of log-phase cultures of *S. Typhimurium* SL1344 WT (left) and induced strain SL1344(pLV1) (right).

$bla_{TEM-1}::kan$) showed similar motility values, either in the absence or the presence of arabinose, in relation to strain *S. Typhimurium* SL1344 WT (data not shown).

Constitutive expression of bla_{VIM-2} in strain SL1344(pSTVIM) was also shown to negatively affect the motility of *S. Typhimurium*, whereas strain SL1344(pST12) displayed behavior similar to that of strain *Typhimurium* SL1344 WT [SL1344(pSTVIM), 16 ± 4.1 mm; SL1344(pST12), 39 ± 7.1 mm; SL1344 WT, 39.3 ± 5.7 mm]. This suggests that the reduction in motility could be associated with the expression of bla_{VIM-2} and not with the plasmid *per se*.

Effect of VIM-2 production on *Salmonella* growth rate. *In vitro* growth rates, measured by changes in OD_{600} , were nearly identical for variants producing the OXA enzyme (induced or uninduced) and strain *S. Typhimurium* SL1344 WT as well [doubling times, SL1344 WT, 25.7 ± 0.6 min; SL1344(pBAD22 $bla_{TEM-1}::kan$), 26.2 ± 1.7 min; induced SL1344(pBAD22 $bla_{TEM-1}::kan$), 25.8 ± 1.2 min; SL1344(pLO5 $bla_{TEM-1}::kan$), 26.4 ± 1.3 min; induced SL1344(pLO5 $bla_{TEM-1}::kan$), 25.9 ± 1 min]. On the other hand, strain SL1344(pLV1 $bla_{TEM-1}::kan$) displayed a lower growth rate, which decreased slightly in the presence of L-arabinose [SL1344(pLV1 $bla_{TEM-1}::kan$), 28.5 ± 0.6 min; induced SL1344(pLV1 $bla_{TEM-1}::kan$), 29.5 ± 0.2 min]. Interestingly, although such differences were not statistically significant, when induced, strain SL1344(pLV1 $bla_{TEM-1}::kan$) reached a stationary growth phase at significantly lower OD_{600} values than the other strains (Fig. 2a). In the absence of induction, strain SL1344(pLV1 $bla_{TEM-1}::kan$) showed a similar behavior to the rest of the strains.

Similar to the results above, constitutive expression of bla_{VIM-2} encoded in plasmid pSTVIM had a biological cost for *Salmonella* in terms of growth rate; OD_{600} values were significantly lower for strain SL1344(pSTVIM) than those obtained for strains SL1344(pST12) and *S. Typhimurium* SL1344 WT (Fig. 2b). Interestingly, after 5 h of incubation, growth curves of strain LVR48 reached a turning point, and after 8 h of incubation the OD_{600} value for this strain was similar to those for SL1344(pST12) and SL1344 WT; this behavior might be partially explained by the loss of the resistance plasmid by strain SL1344(pSTVIM) (Fig. 3).

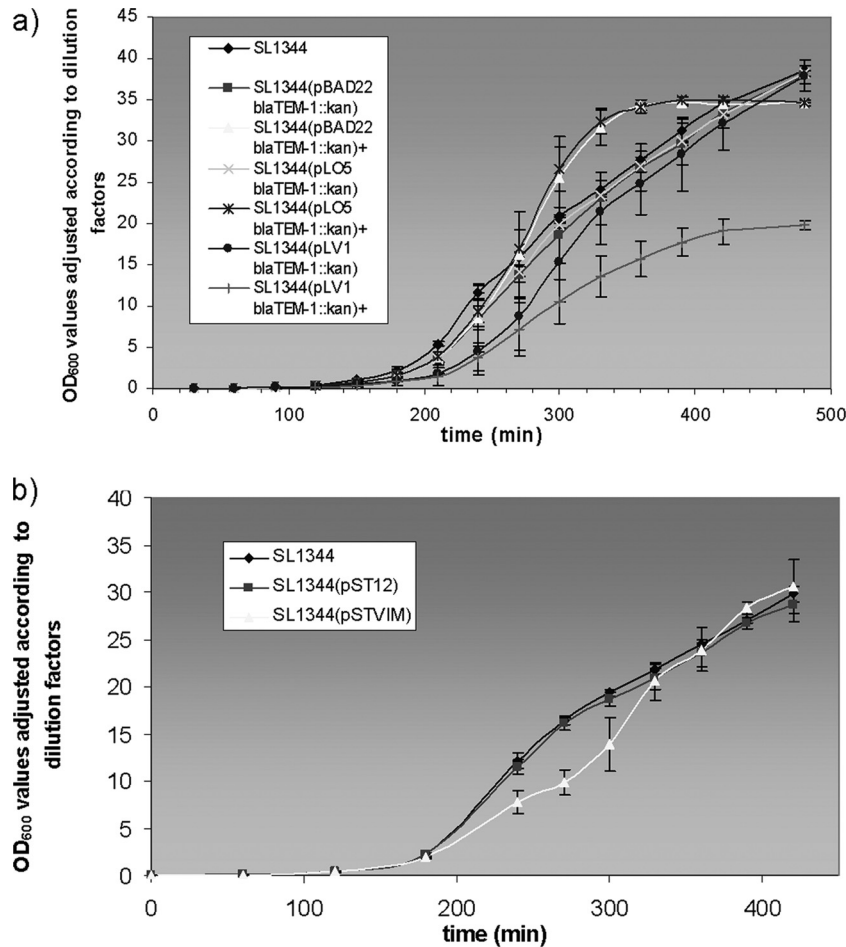


FIG 2 (a) Growth curves for *S. Typhimurium* transformed with the different constructs, in presence and in absence of induction. (b) Growth curves for the constitutive VIM-2-producing strain. In all cases OD₆₀₀ values are adjusted according to the dilutions performed during the assays, and the mean of three independent assays is plotted.

On the other hand, induced and constitutive expression by *E. coli* NF7 of *bla*_{VIM-2} showed results similar to those obtained with *S. Typhimurium* (Fig. 4).

Effect of VIM-2 production on *Salmonella* invasion of epithelial cells. To gain some insight into the influence of *bla*_{VIM-2} expression on the natural life cycle of *Salmonella*, invasion assays were performed in Caco-2 cells. As shown in Fig. 5a, induced synthesis of VIM-2 produced a significant reduction in the invasiveness of strain SL1344(pLV1) compared to the invasiveness of strains carrying *bla*_{OXA-66} or the empty pBAD22 vector or *S. Typhimurium* SL1344 WT. On the other hand, in the absence of L-arabinose, invasion levels for strain SL1344(pLV1) were similar to those of the control strains.

We also determined whether constitutive expression of *bla*_{VIM-2} affected the ability of *S. Typhimurium* to invade epithelial cells. In this sense, strain SL1344(pSTVIM) showed a significant reduction in invasiveness, being approximately 70% less invasive than strain *S. Typhimurium* SL1344 WT (Fig. 5b).

Effect of VIM-2 production on plasmid stability. To assess whether the expression of *bla*_{VIM-2} affected the stability of the plasmid encoding this β -lactamase, strains SL1344(pBAD22 *bla*_{TEM-1}::kan), SL1344(pLO5 *bla*_{TEM-1}::kan), SL1344(pLV1 *bla*_{TEM-1}::kan), SL1344(pST12), and SL1344(pSTVIM) were grown during

ca. 23 generations in the absence of antibiotic selective pressure and in the presence or absence of inducer and then plated on LB agar with and without antibiotic. Under these conditions, plasmids pBAD22 *bla*_{TEM-1}::kan and pLO5 *bla*_{TEM-1}::kan were stably maintained in their respective hosts in either the presence or the absence of inducer.

However, in the absence of antibiotic selective pressure, induced synthesis of VIM-2 determined the loss of plasmid pLV1 *bla*_{TEM-1}::kan in ~90% of the bacterial population, whereas constitutive production of said enzyme (albeit at low levels) resulted in the loss of plasmid pSTVIM in ~25% of the bacterial population, clearly indicating a deleterious effect associated with the production of that MBL (Fig. 3).

DISCUSSION

Although the biological cost of expressing β -lactamases for *Salmonella* has already been studied (14), this work constitutes the first attempt to evaluate the possible alterations associated with MBL production in this important pathogen.

Previously, Morosini et al. showed that the introduction of a plasmidic *ampC* allele into *S. Typhimurium* resulted in a lower growth rate and less invasiveness in MDCK cells (14).

In our work, two separate strategies were followed, controlled

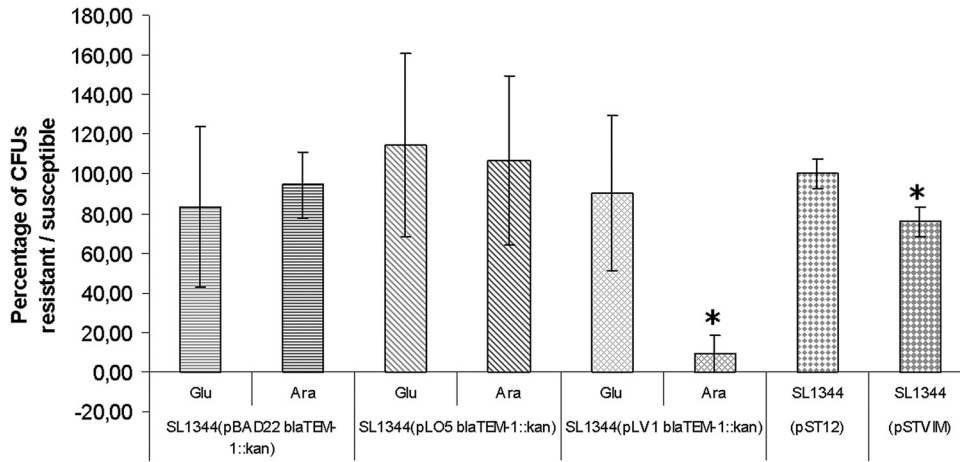


FIG 3 Plasmid stability in the absence of antibiotic selective pressure. The percentage of plasmid-bearing bacteria (kanamycin resistance), in either the absence or presence of inducer, is plotted for each strain. Values over 100% are probably due to technique variations. Asterisks indicate a statistically significant difference in plasmid stability between the repressed and induced states and between the clone carrying pST12 and the clone carrying pSTVIM.

synthesis of VIM-2 by means of an inducible expression vector and weak constitutive production of said enzyme by means of a naturally occurring plasmid obtained from an environmental *S. Derby* isolate.

The different assays in LB media and Caco-2 cells showed a significant fitness reduction for *S. Typhimurium* associated with the production of VIM-2, reflected by a lower growth rate and less motility and invasiveness than the control strains [*S. Typhimurium* SL1344 WT, SL1344(pBAD22), SL1344(pLO5), SL1344(pBAD22 *bla*_{TEM-1}::kan), SL1344(pLO5 *bla*_{TEM-1}::kan), and SL1344(pST12)], as well as alterations in cell and colony morphology. Although these deleterious effects might be related to plasmid maintenance functions, the strain carrying the empty vector (i.e., no insert) as well as that carrying plasmid pLO5 showed similar behavior to *S. Typhimurium* SL1344 WT. Similarly, many

of the phenotypical studies in this work were carried out in the absence of β -lactams, ruling out the possibility of such antibiotics being responsible for the fitness reduction (on account of their effect on the bacterial cell wall).

Our results differ widely from those obtained by Fernández et al. (32). These authors reported that the synthesis of OXA-10-like, OXA-24, and SFO β -lactamases in *E. coli* is accompanied by changes in peptidoglycan composition and a fitness reduction (i.e., lower growth rate in competition assays); on the other hand, these authors found no alterations when *E. coli* expressed VIM-1. Besides the evaluation of different parameters, two possible explanations might account for the discrepancies between this study and the one previously mentioned: (i) differences intrinsic to the microorganisms used as the final receptor (*S. Typhimurium* versus *E. coli*) and to the expression vectors (pBAD22 versus pBGS18-

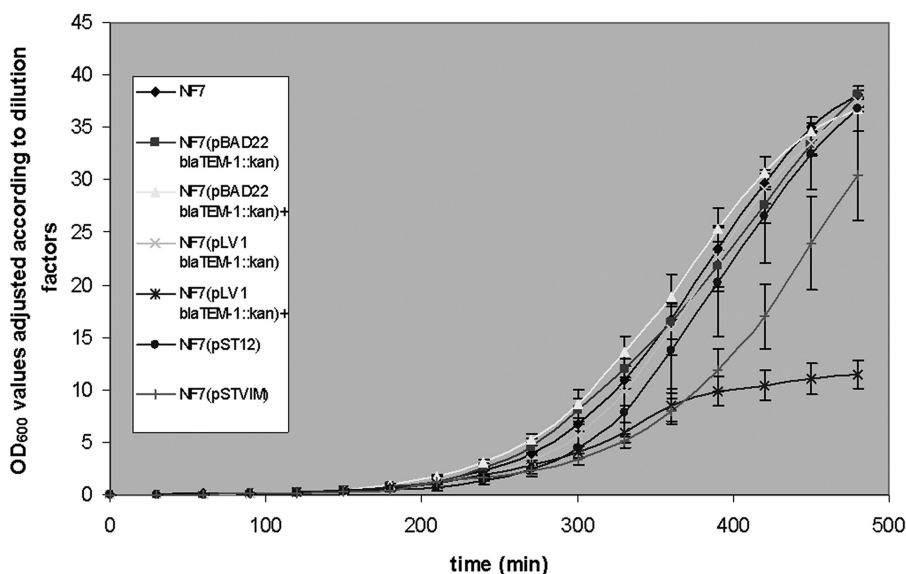


FIG 4 Growth curves in linear scale for *E. coli* strain NF7 transformed with the different constructs for inducible and constitutive VIM-2 production. Plus (+) symbols indicate growth in the presence of inducer. Please note that OD₆₀₀ values are adjusted according to the dilutions performed during the assays. The means of three independent assays are plotted; vertical bars indicate standard deviations.

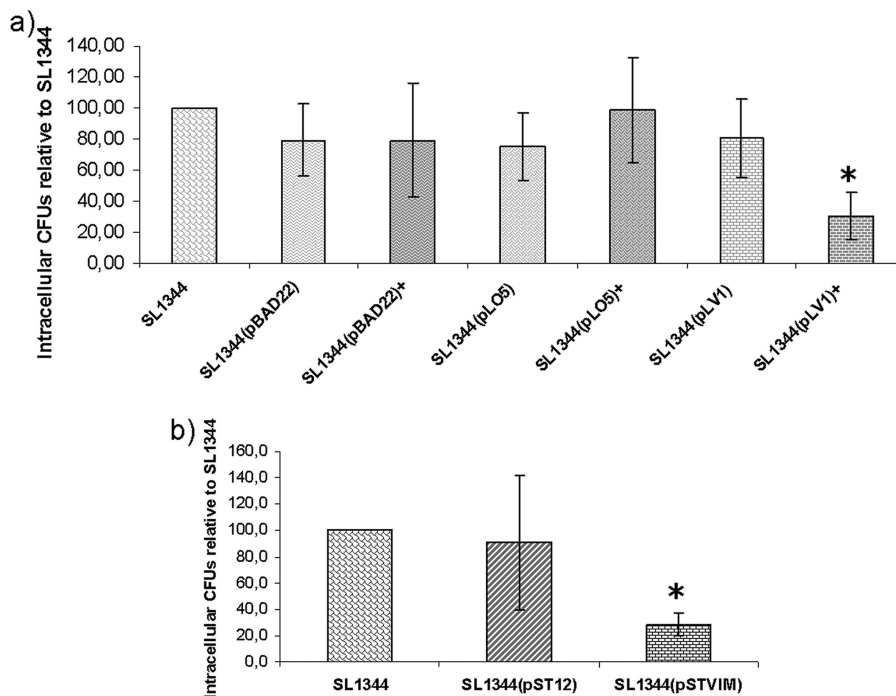


FIG 5 Invasion assays in Caco-2 cells for the inducible VIM-2-producing strain (a) and for the constitutive strain (b). Data are expressed as the means of intracellular CFU of three independent assays, relative to control strain *S. Typhimurium* SL1344. Vertical bars indicate standard deviations. The asterisk indicates a statistically significant difference in invasiveness between the induced or constitutive VIM-2-producing strain and the other strains.

pCT) and (ii) functional properties specific to the enzymes used in each work (VIM-2 versus VIM-1 and OXA-66 versus OXA-24/OXA-10-like). In this regard, the growth curves obtained for VIM-2-producing *E. coli* and *S. Typhimurium* showed similar behaviors, thus suggesting that hypothesis 1 is quite unlikely.

Several factors might be responsible for the fitness reduction for *Salmonella* expressing bla_{VIM-2} . Intuitively, we could adjudicate the loss of motility and invasiveness and the diminished growth rate to saturation of the synthetic pathway due to VIM-2 overproduction. Nevertheless, we observed the same alterations when bla_{VIM-2} was under the control of the weak promoter P_3 (pSTVIM). In addition, bla_{OXA-66} mRNA levels in induced strain SL1344(pLO5) were higher than bla_{VIM-2} levels in strain SL1344(pSTVIM); however, no alterations associated with the former were observed.

Alternatively, Marciano et al. suggested that the signal peptide of the class A carbapenemase SME-1 might be responsible for cell lysis and plasmid rearrangements in an *E. coli* strain, either by forming pores on the bacterial cell wall or by hijacking the protein synthesis machinery (33). Whether the signal peptide of VIM-2 is accountable for the deleterious effects observed in *S. Typhimurium* remains to be investigated.

We report in this work that VIM-2 production, either induced or constitutive (strong and weak promoter, respectively), reduces the ability of *S. Typhimurium* to invade eukaryotic cells. This may be partially explained by the occurrence of filamentous bacteria. The internalization of *Salmonella* in nonphagocytic cells is a highly coordinated event, mediated by flagella, fimbriae, and the SPI-1-encoded type III secretion system (34). Nonmotile mutant salmonellae have been reported to display reduced invasiveness in epithelial cells (35). In this sense, the reduction in the motility of

VIM-2-producing *S. Typhimurium* might also account for the aforementioned loss of invasiveness.

Currently, VIM-2 is the most frequently detected transferable metallo- β -lactamase worldwide (36); nevertheless, there have been only a few reports of the occurrence of VIM enzymes in *Salmonella* isolates.

Furthermore, only a few reports concerning the occurrence of MBL in clinical isolates of this genus have been published; although epidemiologically unrelated, such isolates carried all bla_{NDM-1} (9, 10, 11), and recently, VIM-2. Consequently, the extremely low frequency of class B β -lactamase-producing salmonellae, together with the data presented in this work, support the hypothesis that the expression of such enzymes in this particular microorganism might be accompanied by an important fitness reduction. This biological cost may, in turn, negatively affect the ability of *Salmonella* to colonize the human gut, thus limiting the dissemination of resistant clones. In addition, plasmid stability assays suggest a strong bias toward the loss of the resistant phenotype instead of the development of compensatory mutations alleviating such biological cost; this may also account for the relative worldwide absence of MBL-producing salmonellae.

Our results may partially explain the low frequency of VIM-2-producing salmonellae; however, recent reports of *Salmonella* carrying bla_{NDM} (9, 10, 11) suggest that the latter might impose a lower biological cost on its host. Further studies are required in order to confirm or rule out such a hypothesis.

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