Novel Porin genes and modes of Porin regulation in *Salmonella typhi* S2-1

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Abstrak

Pada S.typhi ompS1 ditemukan gen porin baru yang diduga mempunyai 7 ompR - binding boxes dan 3 promoter. Kami menduga pengaktifan transkripsi, dari P1, dengan Omp R - binding ke boks II dan III (C-type, low affinty); sedangkan boks I dan IV - VII berperan dalam regulasi negatif. Kenyataan bahwa ekspresi Omp S1 meningkat lebih dari 100 kali pada penghilangan dari cis- acting element, yaitu lebih dari 10 kali lipat ekspresi Omp C yang mengkode untuk porin major, menimbulkan pertanyaan yang menarik pada peran dan regulasinya di alam. Analisis ekspresi dari fusi S.typhi dan E. coli Omp C - LacZ, dalam eksperimen cross-complementation baik dengan operon S. typhi atau E. Coli omp B (Omp R / env Z), baik dengan latar belakang S. typhi atau E. coli Omp B, memperlihatkan bahwa keduanya baik S. typhi dan E. coli omp C tidak diregulasi dengan osmolaritas ketika mereka di bawah kontrol S. typhi Omp B dalam S. typhi. Dengan latar belakang S. typhi kedua gen diosmoregulasi di bawah E. coli Omp B. Sebaliknya dalam E. coli mereka berdua di osmoregulasi di bawah S. typhi atau E. coli omp B. Jadi hilangnya osmoregulasi ekspresi S. typhi omp C ditentukan oleh operon S. typhi Omp B dan oleh faktor lain yang tidak diketahui yang terdapat dalam S. typhi.

Abstract

S.typhi ompS1 constitutes a novel porin gene. It has seven putative OmpR-binding boxes and three promoters. Our current model for expression contemplates activation of transcription, from P1, by OmpR-binding to boxes II and III (C-type, low affinity); whereas boxes I and IV-VII would be involved in negative regulation. The fact that ompS1 expression increases more than 100-fold upon removal of the cis-acting elements, and that this level is more than 10-fold higher than that for ompC, which codes for a major porin, raises interesting questions on its role and regulation in nature. The analysis of the expression of S. typhi and E. coli ompC-lacZ fusions, in cross-complementation experiments with either the S.typhi or E. coli omp B (ompR/envZ) operons, in either S.typhi or E.coli ompB backgrounds, showed that both S. typhi and E coli ompC are not regulated by osmolarity when they are under the control of S.typhi ompB in S. typhi. Interestingly, in an S. typhi background , both genes are osmoregulated under E. coli ompB. In contrast, in E.coli, they are both osmoregulated under S. typhi or E.coli ompB. Thus, the lack of osmoregulation of S.typhi ompC expression, is determined both by the S.typhi ompB operon and by other unknown factors present in S.typhi.

Typhoid Fever and other Salmonellosis

Typhoid fever (TF) is still a major health problem throughout the developing world; it is estimated that there are 16.6 million cases annually, with nearly 600 thousand deaths[1,2]. *S. typhi*, the causal agent of TF, causes a septicemia in man; while *S. typhimurium* causes a typhoid-like infection in mice. Thus, new knowledge on *S.typhi* virulence and on the immune response during TF, should allow the design of improved procedures for the control and prevention of the disease, especially in poor areas of the world[3]. Moreover, the new information should be of use towards a global understanding of the molecular pathogenesis of other salmonellosis, on the rise worldwide[2]. Of great relevance will be the possibility of providing new insights on gene regulation in bacteria.

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Porins in *Salmonella typhi*

The bacterial surface structures are of fundamental importance in these processes, as they provide contact with the host cells and milieu. Porins are pore-forming proteins that reside in the outer membrane of gram-negative bacteria; they are believed to participate in the non-selective passive diffusion of small solutes. Accordingly, one might imagine bacteria as having a single-kind of porin, forming multipurpose pores for exchanging nutrients, waste substances, and other compunds, in-and-out of the cell.

Nonetheless, *S.typhi* synthesizes two major porins, OmpC and OmpF, which are highly abundant upon growth in standard laboratory media. It also has a third major outer membrane protein (OMP), denominated OmpA, which is thought to have a structural role[4]. Another major porin, PhoE, is synthesized un-

der phosphate limitation, as its counterpart in *Escherichia coli* (E. Calva et al., unpublished).

In *E.coli*, the pores formed by OmpC and OmpF are weakly cation-selective; in contrast, the PhoE pores are somewhat anion-selective. These three porins produce a similar pore size, although the OmpC pore seems to be slightly smaller, i.e. 1.0 nm versus 1.1-1.2 nm, a characteristic that is thought to confer a selective advantage under high osmolarity, by restricting the diffusion of potentially harmful molecules[5]. In *S. typhimurium,* OmpC, OmpF, and OmpD, form weakly cation-selective pores, relatively larger than the *E.coli* pores, i.e. of 1.2-1.3 nm[6,7]; while no studies on pore size have been done for *S.typhi*. Presently, we know that *S.typhi* OmpC, OmpF, and PhoE contain the conserved amino acid residues that have been implicated in pore function, and that *S.typhi* OmpC and PhoE are 79% and 98 % identical to their *E.coli* counterparts, respectively[8,9], and A. Torres, MSc. Thesis, UNAM, 1995.

Whether the *S. typhi* porins form similar pores to those of their analogues in *E. coli*, is an open question; moreover, the biological significance of having this array of porins is largely unknown. Interestingly, porins appear to play a key role in the permeation of -lactams, as illustrated by an *E. coli* OmpF-deficient mutant with an increased resistance to certain -lactams[7,10].

Immunogenicity of OMPs

A specific humoral and cellular immune response is mounted against *S. typhi* OMPs in TF patients; this has allowed the design of novel diagnostic assays[11-16]. Moreover, an enzyme-linked immunosorbent assay (ELISA) for the rapid and specific detection of TF patients, based on *S. typhi* OMPs and designed for the Mexican population, was also found to be useful in Malaysia[14]. Furthermore, *S. typhimurium* and *S.typhi* OMPs have proven to be relevant immunogens for protection of mice challenged with virulent strains[17-21].

In addition, the protective effect was enhanced when porin-lipopolysaccharide (LPS) complexes were used as immunogens, indicating the role of intricate outer membrane structures in eliciting an immune response [17,21]. These observations might help explain the dependence of the protective effect on the type of LPS present on the surface of the bacteria[22]. In this respect, it has been recently shown that neisserial porins can act synergistically with the LPS, to induce proliferation and immunoglobulin secretion by resting B-cells[23], further supporting the notion that the immune response to the cell envelope is towards a complex mixture of antigens, a subject that requires further addressing by researchers.

Furthermore, the stimulation of peripheral lymphocytes, the activation of complement, and the release of several cytokines, namely tumor necrosis factor-, interleukin-1-, and IL-6, have been observed in response to *S. typhimurium* porins[24-26]. In this regard, immunomodulatory activities, as well as a hypersensitivity reaction, have been recently described for *S. typhi* porins; so that their use as adjuvants or as immunostimulants towards unrelated antigens, such as those from *Mycobacteria*, has been proposed[27].

The Interaction of OMPs with Host Cells

The study of OMPs, including porins, is of relevance to the understanding of the bacterial pathogen-host interaction. Adherence and invasion of mucosal epithelial cells are the first stages in the establishment of infection by enteropathogenic bacteria. Thus, it has been shown that OMPs participate in the invasion of host cells by *Yersinia* and *Shigella*[28-32]. In particular, the lack of OmpC in *Shigella*, results in lowering of virulence due to a decrease in intracellular spreading[31]. Interestingly, *S. typhimurium* strains lacking both the OmpC and OmpF porins have lowered virulence[33]. A *Salmonella* OMP, PagC, has been involved in survival within macrophages[34]. Moreover, it has been found that the *S. typhimurium ompR* porin regulatory locus is of major importance, for the survival of *Salmonella* within host macrophages[35].

Toxic effects by *S. typhimurium* porin preparations on Hela, Hep-2, and mouse renal cells have been observed[24,36]; as well as a stimulation of platelet-activating-factor biosynthesis, by human endothelial cells and polymorphonuclear neutrophils[37].

Regulation of *ompC* in *S.typhi*

Our group has initially focused its research on the major, abundant, *S.typhi* porins and their genes[4,8,39,40], as well as on the *ompR* and *envZ* regulatory loci[41]. In this respect, we have shown that the expression of OmpC in *S.typhi* is not influenced by osmolarity, being always expressed at high levels; whereas expression of OmpC in *E. coli* is indeed higher at high osmolarity. This observation is contrary to the generally accepted view that OmpC has a specific role in high osmolarity environments, such as those found inside

host cells, in the serum, or in the bile tract. On the other hand, OmpF is regulated in the same manner in *S. typhi* as in *E. coli*: it is expressed preferentially at low osmolarity[4].

Our recent observations point towards the existence of particular factors in *S. typhi* that, together with the EnvZ and OmpR regulatory proteins, determine the particular behavior of OmpC expression. The analysis of the expression of *S. typhi* and *E. coli ompC-lacZ* fusions, in cross-complementation experiments with either the *S. typhi* or *E. coli ompB* (*ompR/envZ*) operons, in either *S. typhi* or *E. coli ompB* backgrounds, showed that both *S. typhi* and *E. coli ompC* are not regulated by osmolarity when they are under the control of *S. typhi ompB* in *S. typhi*. Interestingly, in an *S. typhi* background, both genes are osmoregulated under *E. coli ompB*. In contrast, in *E. coli*, they are both osmoregulated under *E.coli ompB*; and, surprisingly, they are also osmoregulated by *S. typhi ompB* (Martínez-Flores, et al, manuscript in preparation).

Thus, the lack of osmoregulation of *S. typhi ompC* expression, is determined both by the *S. typhi ompB* operon and by other unknown factors present in *S. typhi*. Since the OmpR regulator proteins are identical in *S. typhi* and in *E. coli*, it seems likely that this differential behavior is determined, at least in part, by the EnvZ sensor protein, which differs in 21 out of 450 amino acid residues between both bacteria. Interestingly, 16 of these differences lie towards the carboxy-terminus, between residues 303 and 450 [41].

Hence, this project has lured us into probing structure-function relationships in EnvZ, as well as into reflecting on a general and broad question: what is the significance of osmoregulation in bacterial gene expression?

Regulation of *S.typhi ompS1*

Contributing towards an increasingly complex view of the matter, we made the unexpected finding of two novel *S. typhi* porin genes, namely *ompS1* and *ompS2*. This happened while searching for *ompF* by heterologous hybridization with the *E. coli ompF* gene as probe. The *ompS1* and *ompS2* genes code for OMPs with the conserved amino acid motifs, characteristic of enterobacterial and neisserial non-selective porins[9,42]; and are not expressed at major levels under standard laboratory conditions.

By assaying the activity of a reporter gene, linked to various lengths of the *ompS1* 5' regulatory regions, we observed that expression augmented several-fold, reaching and surpassing the levels observed for *ompC*, when defined portions of the regulatory segment were absent, pointing towards regions for the binding and prompt action of negative regulatory elements (Oropeza et al., manuscript in preparation). In addition, expression was not influenced by changes in osmolarity, temperature, pH; nor the presence of polymyxin or $H_2 O_2$; nor by anaerobiosis or growth in DME epithelial culture media (Sampieri et al., unpublished observations). Furthermore, *ompS1* has three promoters: one depends on the OmpR transcriptional activator, and the other two are OmpR-independent; moreover, there are seven putative OmpR-binding boxes in the 5' regulatory region (Orópeza et al., manuscript in preparation).

Our current model for *ompS1* expression contemplates activation of transcription, from P1, by OmpR-binding to boxes II and III (C-type, low affinity); whereas boxes I and IV-VII would be involved in negative regulation. The fact that expression increases more than 100-fold upon removal of the cis-acting elements, and that this level is more than 10-fold higher than that for *ompC*, which codes for a major porin, raises interesting questions on its role and regulation in nature. Thus, we are confronted by a new mode of regulation by OmpR and of porin regulation, in response to unknown physicochemical parameters.

These findings open several questions, such as: why does *Salmonella* require such porin diversity? Is there a yet unknown pore selectivity that warrants such diversity? Moreover, do the OmpS1 and OmpS2 porins constitute relevant antigens for vaccination and diagnosis?

It is particularly intriguing why the *ompS1* and *ompS2* genes are expressed at very low levels under standard laboratory conditions: are the OmpS proteins always expressed at such low levels, and hence fulfill their biological role? or is there a condition, unknown up to now, that allows expression at much higher levels? In addition, we are interested in determining whether the OmpS1 and OmpS2 proteins have a role in the adherence, invasion, and survival of *S. typhi* in tissue culture cells, as well as in the virulence of *Salmonella* in a mouse typhoid model.

Corollary

All these observations evidence the fact that the role of porins and of their regulators, in bacterial physiology, is more complex than initially conceived. Therefore, we are barely starting to pose questions about structure-function relationships and environmental cues, that govern porin expression and function.

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