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Rhodococcus jostii Porin A (RjpA) Functions in Cholate Uptake

Vijayakumar Somalinga,* William W. Mohn

Department of Microbiology and Immunology, Life Sciences Institute, University of British Columbia, Vancouver, BC, Canada

RjpA in *Rhodococcus jostii* is the ortholog of a channel-forming porin, MspA. Deletion of *rjpA* delayed growth of *R. jostii* on cholate but not on cholesterol. Eventual growth on cholate involved increased expression of other porins, namely, RjpB, RjpC, and RjpD. Porins appear essential for the uptake of bile acids by mycolic acid bacteria.

Steroids are important biomolecules occurring in all domains of life. Steroids are ubiquitous in the environment as a result of excretion and biomass decomposition, as well as industrial and municipal waste discharges. These steroids can be detrimental to both humans (1) and ecosystems (2). Some bacteria degrade steroids, and a few pathways permitting catabolism of steroids have been partially elucidated (3–6). Recent studies have also indicated that steroid uptake and metabolism play important roles in the virulence of both human (7, 8) and animal (9) pathogens. Despite the importance of steroids and their transformation by microorganisms, there remain substantial gaps in our knowledge of microbial steroid uptake and metabolism.

Rhodococcus jostii is a soil-dwelling, metabolically versatile member of the mycolic acid-containing actinobacteria that grow on a range of steroids (10). BLAST analysis of the *R. jostii* genome revealed four proteins, RjpA (Ro04074), RjpB (Ro03127), RjpC (Ro03156), and RjpD (R08561), with 33, 33, 31, and 36% identities, respectively, to a channel-forming porin from *Mycobacterium smegmatis* known as MspA (*Mycobacterium smegmatis* porin A). Furthermore, the presence of signal peptide cleavage sites in RjpA, RjpB, and RjpD indicates that these proteins, like MspA, target the membrane. RjpA and MspA are reciprocal best BLAST hits, indicating that they are orthologous. A structural homology search using RjpA as a query also retrieved MspA, suggesting that RjpA forms a channel similar to that formed by MspA. The MspA monomers oligomerize to form a large (>100-kDa) homo-octameric, goblet-shaped protein with a central pore spanning the outer membrane (11). Loop regions of the protein lining the pore eyelet undergo conformational change, which may affect the uptake of ions and solutes by the porin (12). In *M. smegmatis*, MspA is involved primarily in the uptake of hydrophilic solutes, such as glucose (13) and phosphates (14). MspA has been shown to be the main conduit for hydrophilic antibiotics, such as fluoroquinolones and chloramphenicol (15), in addition to nutrients. Deletion of MspA also caused a marked increase in resistance to hydrophobic antibiotics, such as rifampin and erythromycin (16). Furthermore, deletion of MspA resulted in a 3-fold reduction in the uptake of the bile acid steroid chenodeoxycholate (16).

The similarity of RjpA to the MspA porin and the effect of MspA deletion on the uptake of hydrophobic antibiotics and a steroid led to the hypothesis that RjpA is involved in the uptake of steroids by *R. jostii*, which this study investigated.

The *rjpA* gene was deleted from *R. jostii*, and the mutant was tested for its ability to grow on cholesterol or cholate. The *rjpA* gene of *R. jostii* was completely deleted, in frame and unmarked, using the *sacB* counterselection system described previously (17) and primers in Table 1. Deletion of *rjpA* did not affect the growth of *R. jostii* on cholesterol (Fig. 1). This result suggests that RjpA is not involved in the uptake of highly hydrophobic steroids, such as cholesterol. Both *R. jostii* and *Mycobacterium tuberculosis* take up cholesterol and, in the case of *R. jostii*, also β-sitosterol, via the Mce4 system, an unusually complex ATP-binding cassette transporter (7, 10). Functionally essential components of the Mce4 system include putatively extracytoplasmic Mce proteins, which we speculate may facilitate movement of hydrophobic steroids across the outer membrane and periplasm. The Mce4 system was not required for the uptake of, and growth on, cholate. In contrast to results with cholesterol, growth of the *rjpA* mutant on the more hydrophilic bile acid cholate was impaired. Initiation of growth of the mutant on cholate was 2 days later than that of the wild type (Fig. 2). Subsequently, the mutant grew at the same rate and to the same final cell density as the wild type. The 2-day difference in growth initiation was highly reproducible in independent experiments.

To verify that deletion of *rjpA* caused delayed growth on cholate, the mutation was complemented. The *rjpA* gene was cloned into the pTip-QC2 vector and expressed in the *rjpA* mutant strain, using previously described methods (10) and primers in Table 1. This complementation completely restored the wild-type growth phenotype on cholate (Fig. 2).

The ability of the *rjpA* mutant, following the 2-day delay, to grow on cholate at the same rate and to the same final density as the wild type suggested that *R. jostii* may compensate for the *rjpA* deletion by upregulating other porins. Three paralogs of *rjpA*, *ro03127* (*rjpB*), *ro03156* (*rjpC*), and *ro08561* (*rjpD*), were identified by BLAST search. To determine whether the other *rjp* porins might compensate for the *rjpA* deletion, levels of expression of all four porin genes during exponential growth (optical density at 600 nm [OD_{600}] = 0.8) were compared using reverse transcriptase quantitative-PCR (RT-QPCR). The wild type expressed *rjpA* at much higher levels than the other porin genes on both pyruvate and cholate (Table 2), indicating that under our growth conditions, RjpA is the major porin in *R. jostii*. Compared to the wild type, the mutant greatly increased expression of *rjpB*, *rjpC*, and

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Address correspondence to William W. Mohn, wmohn@mail.ubc.ca.
* Present address: Vijayakumar Somalinga, Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, California, USA.
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TABLE 1 Primers and probes used in this study

No.	Primer	Sense or probe	5′–3′ sequence[a]	Function
1	*ro04074*	Forward	TGG TCA GGA TGC AGG GAG TT	Primers and probes for quantification of porin genes by RT-QPCR
2	*ro04074*	Probe *rpJA*	TGG CTC CAT CTC C	
3	*ro04074*	Reverse	GTG ACC GGC CCG ATA GC	
4	*ro03127*	Forward	GAG GCA ACC GGG TCG AA	
5	*ro03127*	Probe *rpJB*	TCC TGC AAG GTG ATA CC	
6	*ro03127*	Reverse	CGG CGG GAC CAC TTG ATA C	
7	*ro03156*	Forward	CAG TGG CTC AAC GAC GTG AT	
8	*ro03156*	Probe *rpJC*	CTC AAC GGC ACC CC	
9	*ro03156*	Reverse	TGG ACT TGA TCG CGG TGT T	
10	*ro08561*	Forward	CCG CCG CTG GAT GGT	
11	*ro08561*	Probe *rpJD*	TTC CCA CCA GTG TCG AG	
12	*ro08561*	Reverse	GGC GTA ACC GTT GTG GAA GA	
13	*ro04074*_P1	Forward	CCC<u>AAGCTT</u>AGGCGATCAGCAAGCCGAGGACG	Upstream flanking region amplification primers
14	*ro04074*_P2	Reverse	TGC<u>TCTAGA</u>CTGCGACTGCCACCGAGCGTG	
15	*ro04074*_P3	Forward	TGC<u>TCTAGA</u>GGCCGTGACGGAGAACGGTG	Downstream flanking region amplification primers
16	*ro04074*_P4	Reverse	CGC<u>GGATCC</u>CGGTGTGCTGGGCGCG	
17	*ro04074*_P7	Forward	CGGATTCGAGGAGCGGCAGGTG	*rjpA* deletion confirmation primers
18	*ro04074*_P8	Reverse	GGCGGTGAGGTGGTG GTGCATG	
19	*ro04074*_P9	Forward	GGAATTC<u>CATATG</u>GGTCGGCATGACGGACATCAGC	Primers to amplify *rjpA* for complementation
20	*ro04074*_P10	Reverse	CCC<u>AAGCTT</u>CTAGTTGAGCTTCTGCGGCACACCG	

[a] Restriction sites are underlined.

rjpD during growth on cholate, by 45-, 65-, and 26-fold, respectively. In contrast, on pyruvate, the *rjpA* mutant grew normally and did not increase expression of *rjpB*, *rjpC*, and *rjpD*, relative to that of the wild type. Thus, despite its high level of expression, RjpA does not play a critical role in growth on pyruvate. Expression of *sigA* is expected to be correlated with the cellular growth rate, and it did not vary by more than 2-fold among the assays. This indicates that the large differences in expression of *rjp* genes observed are not attributable to major differences in growth rates among the two strains on the two substrates.

Overall, the results indicate that porins are essential for efficient cholate uptake. Of the porins, RjpA plays the main role in cholate uptake, but at least one of the other three porins can also serve that function and compensate for the loss of RjpA. A similar phenomenon was observed in *M. smegmatis* growing on glycerol (18). The *mspA* gene codes for the most highly expressed porin in *M. smegmatis*. Only *mspA* and, to a much lesser extent, *mspC* were expressed in wild-type *M. smegmatis* (18). In response to the deletion of *mspA*, expression of *mspB* and *mspD* was increased. Moreover, the deletion of *mspA* resulted in a significant decrease of nutrient permeability across the outer membrane, while the deletion of other porin genes caused no significant reduction in nutrient uptake (18).

We are beginning to understand steroid uptake by bacteria. This and other studies suggest that different uptake mechanisms are employed for the most hydrophobic steroids versus the more hydrophilic bile acids. So far, three systems that transport bile acids across the cytoplasmic membrane have been characterized. One is the well-characterized BaiG protein, a 50-kDa integral membrane protein from *Eubacterium* sp. strain VP1 12708 (19). BaiG cloned into *Escherichia coli* has been shown to transport unconjugated cholate and chenodeoxycholate (19). The second bile acid transporter was identified in *Lactobacillus johnsonii* and cloned into *E. coli* to demonstrate its ability to transport cholate

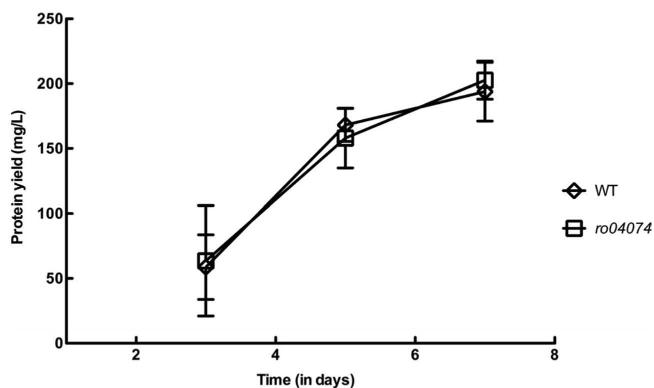

FIG 1 Growth of *R. jostii* (wild type [WT]) and the *rjpA* (Δ*ro04074*) mutant on cholesterol ($n = 3$; bars indicate standard errors). Cultures were grown on defined medium as previously described (22), with 2.0 mM cholesterol as the sole organic substrate. Because precipitated cholesterol interfered with optical density measurement, growth was measured as protein using the bicinchoninic acid (BCA) assay (Pierce) after hot alkaline lysis.

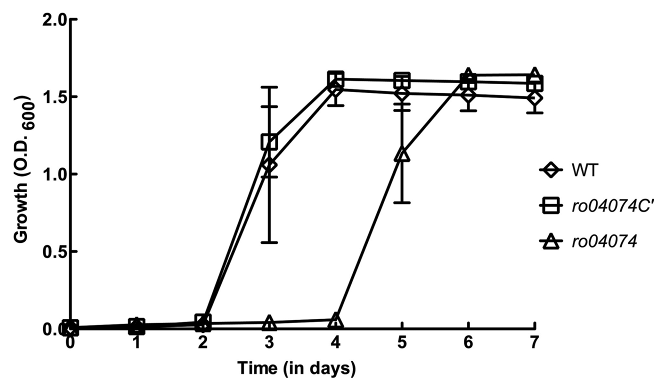

FIG 2 Growth of *R. jostii* (WT), the *rjpA* mutant (Δ*ro04074*), and the *rjpA* complementation strain (Δ*ro04074C*) on cholate ($n = 3$; bars indicate standard errors). Growth conditions were as described for Fig. 1, except that 2.0 mM cholate was the sole organic substrate.

TABLE 2 Abundance of porin transcripts during exponential growth of *R. jostii* or the *rjpA* mutant strain on either pyruvate or cholate

	No. of gene transcripts (no. of copies/ng of DNA) after growth on[a]:			
	Pyruvate (20 mM)		Cholate (2 mM)	
Gene	*R. jostii*	Mutant	*R. jostii*	Mutant
rjpA	445,800 ± 195, 800	250 ± 160	28,300 ± 1,860	4 ± 4
rjpB	800 ± 320	180 ± 9	35 ± 4	1,500 ± 440
rjpC	50 ± 5	30 ± 1	22 ± 1	1,400 ± 140
rjpD	1,330 ± 240	350 ± 32	144 ± 39	3,800 ± 290
sigA	15,800 ± 330	30,800 ± 7,200	21,000 ± 720	27,300 ± 2,500

[a] Values are means ± standard deviation ($n = 3$). Transcripts were measured by reverse transcriptase quantitative-PCR, as described previously (23), using the primers and probes in Table 1.

(20). Recently, a transporter from *Neisseria meningitidis* homologous to the human apical sodium-dependent bile acid transporter (ASBT) was structurally characterized (21). The current study advances our understanding of the role of porins in transport of a bile acid across an outer membrane. Stephan et al. (16) proposed that the presence of porins might affect rates of diffusion of chenodeoxycholate through the lipids of the outer membrane of *M. smegmatis*. However, based on the essentiality of porins for growth of RHA1 on cholate, a more parsimonious conclusion may be that bile acids diffuse through porin channels. Clearly, further biochemical and structural studies are needed to elucidate the mechanism by which RjpA and other porins facilitate the uptake of bile acids. It is likely that bile acid uptake by mycolic acid bacteria, in general, requires porins. It is further possible that bile acid uptake by Gram-negative bacteria also requires porins, as their outer membranes are structurally analogous to those of mycolic acid bacteria.

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