Short-Form paper:

Rhodococcus jostii porin A, RjpA, functions in cholate uptake

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Running title: Rhodococcus jostii porin A, RjpA, RjpA functions in cholate uptake
Abstract:

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. Eventually growth on cholate involved increased expression of other porins, RjpB, RjpC and RjpD. Porins appear essential for the uptake of bile acids by mycolic acid bacteria.

Main Text

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 pathogens (9). 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 mycolic acid containing Actinobacterium that grows 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 % identity to a channel-forming porin from *Mycobacterium smegmatis* known as MspA (*Mycobacterium smegmatis* porin A) respectively. Furthermore, the presence of signal peptide cleavage site in RjpA,
RjpB and RjpD indicates that these proteins targeted to the membrane similar to MspA. 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 of 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 primarily involved in the uptake of hydrophilic solutes such as glucose (13) and phosphates (14). In addition to nutrients, MspA has also been shown to be the main conduit for hydrophilic antibiotics such as fluoroquinolones and chloramphenicol (15). 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 may be 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 un-marked, using the * sacB* counter selection system as described previously (17) and primers in Table 1. Deletion of *rjpA* did not affect 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 *M. tuberculosis* uptake
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 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 two 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 two-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 two-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 up regulating 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, expression of all four porin genes during exponential growth (OD$_{600}$ = 0.8) was compared, using reverse-transcriptase quantitative-PCR. 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 *rjpD* during growth on cholate, 45-, 65- and 26-fold, respectively. By contrast, on pyruvate, the *rjpA* mutant grew normally and did not increase expression of *rjpB*, *rjpC* and *rjpD*, relative to the wild type. Thus, despite its high expression level, 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 two 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 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, a 50-kDa integral membrane protein from *Eubacterium sp.*
strain VP1 12708 (19). BaiG cloned into *E. 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 (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 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.

**Acknowledgements**

We thank Jie Liu for providing RHA1 genomic DNA and Gordon Steward for technical
assistance. This research was funded by a CIHR Operating Grant.
References


**Figure legends**

Figure 1: Growth of *R. jostii* (WT) and the *rjpA* mutant (Δro04074) on cholesterol (n = 3; bars indicate standard error). 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 BCA assay (Pierce) after hot alkaline lysis.

Figure 2: Growth of *R. jostii* (WT), the *rjpA* mutant (Δro04074) and the *rjpA* complementation strain (Δro04074C) on cholate (n = 3; bars indicate standard error). Growth conditions were as in Fig. 1, except that 2.0 mM cholate was the sole organic substrate.
Table 1: Primers and probes used in this study. Restriction sites are underlined.

<table>
<thead>
<tr>
<th>#</th>
<th>Primers</th>
<th>Sense</th>
<th>5’-3’ sequence</th>
<th>Function</th>
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<tbody>
<tr>
<td>1</td>
<td>ro04074</td>
<td>Forward</td>
<td>TGG TCA GGA TGC AGG GAG TT</td>
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<td>2</td>
<td>ro04074</td>
<td>Probe rjA</td>
<td>TGG CTC CAT CTC C</td>
<td>Primers and probes used for quantification of porin genes using RT-QPCR</td>
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<tr>
<td>3</td>
<td>ro04074</td>
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<td>GTG ACC GGC CCG ATA GC</td>
<td></td>
</tr>
<tr>
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<td>ro03127</td>
<td>Forward</td>
<td>GAG GCA ACC GGG TCG AA</td>
<td></td>
</tr>
<tr>
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<td>ro03127</td>
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<td>TCC TGC AAG GTG ATA CC</td>
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<td>CTC AAC GGC ACC CC</td>
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<td>CGGATCCAGGAGGAGGACGGT</td>
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Table 2: Abundance of porin transcripts during exponential growth of *R. jostii* or the *rjpA* mutant strain on either pyruvate or cholate* (n=3; values are means ± standard deviation)

<table>
<thead>
<tr>
<th>Genes</th>
<th>Pyruvate (20 mM)</th>
<th>Cholate (2 mM)</th>
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<tr>
<td></td>
<td><em>R. jostii</em></td>
<td>Mutant</td>
</tr>
<tr>
<td><em>rjpA</em></td>
<td>445,800 ± 195, 800</td>
<td>250 ± 160</td>
</tr>
<tr>
<td><em>rjpB</em></td>
<td>800 ± 320</td>
<td>180 ± 9</td>
</tr>
<tr>
<td><em>rjpC</em></td>
<td>50 ± 5</td>
<td>30 ± 1</td>
</tr>
<tr>
<td><em>rjpD</em></td>
<td>1330 ± 240</td>
<td>350 ± 32</td>
</tr>
<tr>
<td><em>sigA</em></td>
<td>15,800 ± 330</td>
<td>30,800 ± 7200</td>
</tr>
</tbody>
</table>

*Transcripts were measured by reverse-transcriptase quantitative-PCR, as described previously (23), using primers and probes in Table 1.
Figure 1: Growth of *R. jostii* (WT) and the *rjpA* mutant (Δro04074) on cholesterol (n = 3; bars indicate standard error). 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 BCA assay (Pierce) after hot alkaline lysis.
Figure 2: Growth of *R. jostii* (WT), the *rjpA* mutant (Δro04074) and the *rjpA* complementation strain (Δro04074C') on cholate (n = 3; bars indicate standard error).

Growth conditions were as in Fig. 1, except that 2.0 mM cholate was the sole organic substrate.