

# Rhamnolipids: diversity of structures, microbial origins and roles

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**Abstract** Rhamnolipids are glycolipidic biosurfactants produced by various bacterial species. They were initially found as exoproducts of the opportunistic pathogen *Pseudomonas aeruginosa* and described as a mixture of four congeners:  $\alpha$ -L-rhamnopyranosyl- $\alpha$ -L-rhamnopyranosyl- $\beta$ -hydroxydecanoyl- $\beta$ -hydroxydecanoate (Rha-Rha-C<sub>10</sub>-C<sub>10</sub>),  $\alpha$ -L-rhamnopyranosyl- $\alpha$ -L-rhamnopyranosyl- $\beta$ -hydroxydecanoate (Rha-Rha-C<sub>10</sub>), as well as their mono-rhamnolipid congeners Rha-C<sub>10</sub>-C<sub>10</sub> and Rha-C<sub>10</sub>. The development of more sensitive analytical techniques has led to the further discovery of a wide diversity of rhamnolipid congeners and homologues (about 60) that are produced at different concentrations by various *Pseudomonas* species and by bacteria belonging to other families, classes, or even phyla. For example, various *Burkholderia* species have been shown to produce rhamnolipids that have longer alkyl chains than those produced by *P. aeruginosa*. In *P. aeruginosa*, three genes, carried on two distinct operons, code for the enzymes responsible for the final steps of rhamnolipid synthesis: one operon carries the *rhlAB* genes and the other *rhlC*. Genes highly similar to *rhlA*, *rhlB*, and *rhlC* have also been found in various *Burkholderia* species but grouped within one putative operon, and they have been shown to be required for rhamnolipid production as well. The exact physiological function of these secondary metabolites is still unclear. Most identified activities are derived from the surface activity, wetting ability, detergency, and other amphipathic-related properties of these molecules. Indeed, rhamnolipids promote the uptake and biodegradation of poorly soluble substrates,

act as immune modulators and virulence factors, have antimicrobial activities, and are involved in surface motility and in bacterial biofilm development.

**Keywords** Biosurfactants · *Pseudomonas* · *Burkholderia*

## Introduction

Surfactants are chemical compounds that (as entailed by their name) display surface activity. They have predilection for interfaces of dissimilar polarities (liquid–air or liquid–liquid) and are soluble in both organic (non-polar) and aqueous (polar) solvents. This property originates from their amphiphilic (or amphipathic) structures, which comprise both hydrophilic (head) and hydrophobic groups (tail) (Desai and Banat 1997). Biosurfactants are surfactants of biological origin. Microorganisms like bacteria, yeasts, and fungi are known to produce various types of biosurfactants. Their hydrophilic part is usually composed of sugars, amino acids, or polar functional groups like carboxylic acid groups. The hydrophobic part is typically an aliphatic hydrocarbon chain of  $\beta$ -hydroxy fatty acids (Lang and Wullbrandt 1999). Surfactants are generally classified according to the charge carried by their polar groups (head) into cationic, anionic, amphoteric, and nonionic.

Biosurfactants are attracting much attention because they represent ecological alternatives to their synthetic counterparts: they exhibit lower toxicity, potentially high activities, and stability at extremes of temperature, pH, and salinity. They have a wide variety of structures, and they can be produced from renewable feedstocks by a wide variety of microorganisms. Most importantly, they are biodegradable, making them environmentally friendly, “green” chemicals.

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Biosurfactants are classified according to their molecular structure into mainly glycolipids (e.g., rhamnolipids (RLs) and sophorolipids), lipopeptides (e.g., surfactin), polymeric biosurfactants (e.g., emulsan and alasan), fatty acids (e.g., 3-(3-hydroxyalkanoyloxy)alkanoic acids (HAAs)), and phospholipids (e.g., phosphatidylethanolamine) (Desai and Banat 1997; Lang and Wullbrandt 1999).

Rhamnolipids are surface-active glycolipids. They have been intensively investigated and extensively reviewed (Maier and Soberón-Chávez 2000; Nitschke et al. 2005; Ochsner et al. 1996; Soberón-Chávez 2004; Soberón-Chávez et al. 2005). However, two questions about this class of biosurfactants are emerging as important topics that have yet been poorly reviewed. First, what is the intrinsic, natural role of RLs for the producing organisms? Second, what actually are these producers? Indeed, while the opportunistic pathogen *Pseudomonas aeruginosa* has traditionally been considered the primary RL-producing microorganism, many other bacterial species, especially in recent years, have been reported to produce RLs as well. Still, apart from some clear cases, it appears that the ability to produce RLs is in fact restricted to a limited number of species, and that many reports are anecdotal. Although initially only four RL species had been described, the development of more sensitive analytical techniques has revealed the co-production of a far wider variety of RL congeners. Thus, this review focuses on the diversity of reported RL bacterial producers, the chemical structure of identified RLs, and the various physiological functions and roles attributed to RLs for the producing bacteria.

#### Diversity of chemical structures of rhamnolipids

The initial discovery of RLs goes back to 1946 when Bergström et al. (1946a, b) reported an oily glycolipid produced by *Pseudomonas pyocyanea* (now *P. aeruginosa*) after growth on glucose that was named pyolipic acid and whose structural units were identified as L-rhamnose and  $\beta$ -hydroxydecanoic acid (Hauser and Karnovsky 1954; Jarvis and Johnson 1949). Jarvis and Johnson (1949) further elucidated the structure of a RL isolated from *P. aeruginosa* and showed that it was composed of two  $\beta$ -hydroxydecanoic acids linked through a glycosidic bond to two rhamnose moieties, with the two  $\beta$ -hydroxy fatty acid portions linked through an ester bond while the disaccharide portion contained a putative 1,3-glycosidic linkage. Edwards and Hayashi (1965) rather found that the linkage between the two rhamnose moieties is an  $\alpha$ -1,2-glycosidic linkage, as determined by periodate oxidation and methylation. Based on that, they chemically described this RL as 2-O- $\alpha$ -1,2-L-rhamnopyranosyl- $\alpha$ -L-rhamnopyranosyl- $\beta$ -hydroxydecanoyl- $\beta$ -hydroxydecanoate (di-RL, structure 39 in Table 1). This was the first discovered

glycolipid containing a link between a sugar and a hydroxylated fatty acid residue (Shaw 1970).

Later on, Itoh et al. (1971) isolated and identified a new RL congener produced concurrently with the aforementioned di-RL by several strains of *P. aeruginosa* after growth on *n*-paraffin, and it was identified as  $\alpha$ -L-rhamnopyranosyl- $\beta$ -hydroxydecanoyl- $\beta$ -hydroxydecanoate (mono-RL, structure 13 in Table 1). Moreover, they postulated that this mono-RL is the precursor of the di-RL (Itoh et al. 1971), although this had been suggested previously (Burger et al. 1963). A few years later, two additional RL congeners were identified in cultures of *Pseudomonas* sp. DSM 2874 grown as resting cells on *n*-alkanes or glycerol (Syldatk et al. 1985). These were similar to the previously identified mono- and di-RLs but containing only one  $\beta$ -hydroxydecanoic acid side chain (mono-lipidic RLs; structures 3 and 31 in Table 1). These mono-lipidic RLs were also detected in trace concentrations in continuous *P. aeruginosa* cultures (Gruber et al. 1993). Nevertheless, it is unlikely that these mono-lipidic congeners require special culture conditions to be produced since many recent studies have identified the four RL congeners produced by various *P. aeruginosa* in batch-mode production. For instance, Arino et al. (1996) have reported the following composition in a *P. aeruginosa* batch culture: 67% di-rhamno-di-lipid, 22% mono-rhamno-di-lipid, 9% di-rhamno-mono-lipid, and less than 3% of mono-rhamno-mono-lipid.

Furthermore, two unusual di-lipidic RLs have been identified with a further acylation with a 2-decenoyl group at the free 2-hydroxyl group of the rhamnose moieties, in cultures of a hydrocarbon-utilizing *Pseudomonas* sp. after growth on *n*-paraffin (structures 29 and 58 in Table 1; Yamaguchi et al. 1976). These two congeners have been also described by Lang and Wagner (1987). Since then, however, very few teams have reported these two congeners, a possible indication that they are strain-specific (Ishigami et al. 1987a, b, 1996; Yamaguchi et al. 1978). Finally, two novel RLs were described as methyl esters of the free carboxylic groups of two previously identified di-lipidic RLs (structures 28 and 57 in Table 1; Hirayama and Kato 1982). These two methylated congeners are the only known nonionic RLs.

Apart from these eight RL congeners identified until the mid-1980s, the last decade of the twentieth century has witnessed the discovery of a large number of new RLs that paralleled the implementation of more sensitive analytical

Many of the listed homologues were identified in trace amounts. The designation C<sub>x,1</sub> means a fatty acid chain with chain length of X and with one unsaturated bond (–2H). The designation C<sub>x,2</sub> means a fatty acid chain with chain length of X and with two unsaturated bonds (–4H).

*Rha* is an  $\alpha$ -L-rhamnosyl moiety, *b* is an  $\alpha$ -decenoyl moiety drawn against mono-rhamno-di-lipidic congeners, *d* is the structure of the triacylated di-RL produced by *Burkholderia plantarii* (Andră et al. 2006)

<sup>a</sup>The exact order of the fatty acid chains has not been definitively determined by the authors (Gunther et al. 2005)

**Table 1** Chemical structure of different identified rhamnolipid congeners and homologues

No.	Symbol	M. Form.	MW	R <sub>1</sub>	n <sub>1</sub>	n <sub>2</sub>	R <sub>2</sub>	Structure	
<b>Mono-rhamno-mono-lipidic congeners</b>									
1	Rha-C <sub>8;2</sub>	C <sub>14</sub> H <sub>22</sub> O <sub>7</sub>	302.32	H	1(-4H)	-	H		
2	Rha-C <sub>8</sub>	C <sub>14</sub> H <sub>26</sub> O <sub>7</sub>	306.35	H	1	-	H		
3	Rha-C <sub>10</sub>	C <sub>16</sub> H <sub>30</sub> O <sub>7</sub>	334.41	H	3	-	H		
4	Rha-C <sub>12;2</sub>	C <sub>18</sub> H <sub>30</sub> O <sub>7</sub>	358.43	H	5(-4H)	-	H		
5	Rha-C <sub>12</sub>	C <sub>18</sub> H <sub>34</sub> O <sub>7</sub>	362.46	H	5	-	H		
6	Rha-C <sub>14;2</sub>	C <sub>20</sub> H <sub>34</sub> O <sub>7</sub>	386.48	H	7(-4H)	-	H		
<b>Mono-rhamno-di-lipidic congeners</b>									
7	Rha-C <sub>8</sub> -C <sub>8</sub>	C <sub>22</sub> H <sub>40</sub> O <sub>9</sub>	448.55	H	1	1	H		
8	Rha-C <sub>8</sub> -C <sub>10;1</sub>	C <sub>24</sub> H <sub>42</sub> O <sub>9</sub>	474.58	H	1	3(-2H)	H		
9	Rha-C <sub>10;1</sub> -C <sub>8</sub>	C <sub>24</sub> H <sub>42</sub> O <sub>9</sub>	474.58	H	3(-2H)	1	H		
10	Rha-C <sub>8</sub> -C <sub>10</sub>	C <sub>24</sub> H <sub>44</sub> O <sub>9</sub>	476.60	H	1	3	H		
11	Rha-C <sub>10</sub> -C <sub>8</sub>	C <sub>24</sub> H <sub>44</sub> O <sub>9</sub>	476.60	H	3	1	H		
12	Rha-C <sub>10</sub> -C <sub>10;1</sub>	C <sub>26</sub> H <sub>46</sub> O <sub>9</sub>	502.64	H	3	3(-2H)	H		
13	Rha-C <sub>10</sub> -C <sub>10</sub>	C <sub>26</sub> H <sub>48</sub> O <sub>9</sub>	504.65	H	3	3	H		
14	Rha-C <sub>8</sub> -C <sub>12</sub>	C <sub>26</sub> H <sub>48</sub> O <sub>9</sub>	504.65	H	1	5	H		
15	Rha-C <sub>12</sub> -C <sub>8</sub>	C <sub>26</sub> H <sub>48</sub> O <sub>9</sub>	504.65	H	5	1	H		
16	Rha-C <sub>10</sub> -C <sub>12;1</sub>	C <sub>28</sub> H <sub>50</sub> O <sub>9</sub>	530.69	H	3	5(-2H)	H		
17	Rha-C <sub>12;1</sub> -C <sub>10</sub>	C <sub>28</sub> H <sub>50</sub> O <sub>9</sub>	530.69	H	5(-2H)	3	H		
18	Rha-C <sub>10</sub> -C <sub>12</sub>	C <sub>28</sub> H <sub>52</sub> O <sub>9</sub>	532.71	H	3	5	H		
19	Rha-C <sub>12</sub> -C <sub>10</sub>	C <sub>28</sub> H <sub>52</sub> O <sub>9</sub>	532.71	H	5	3	H		
20	Rha-C <sub>10</sub> -C <sub>14;1</sub> <sup>a</sup>	C <sub>30</sub> H <sub>54</sub> O <sub>9</sub>	558.74	H	3	7(-2H)	H		
21	Rha-C <sub>12</sub> -C <sub>12;1</sub> <sup>a</sup>	C <sub>30</sub> H <sub>54</sub> O <sub>9</sub>	558.74	H	5	5(-2H)	H		
22	Rha-C <sub>10</sub> -C <sub>14</sub> <sup>a</sup>	C <sub>30</sub> H <sub>56</sub> O <sub>9</sub>	560.76	H	3	7	H		
23	Rha-C <sub>12</sub> -C <sub>12</sub>	C <sub>30</sub> H <sub>56</sub> O <sub>9</sub>	560.76	H	5	5	H		
24	Rha-C <sub>12</sub> -C <sub>14</sub>	C <sub>32</sub> H <sub>60</sub> O <sub>9</sub>	588.81	H	5	7	H		
25	Rha-C <sub>14</sub> -C <sub>14</sub>	C <sub>34</sub> H <sub>64</sub> O <sub>9</sub>	616.87	H	7	7	H		
26	Rha-C <sub>14</sub> -C <sub>16</sub>	C <sub>36</sub> H <sub>68</sub> O <sub>9</sub>	644.92	H	7	9	H		
27	Rha-C <sub>16</sub> -C <sub>16</sub>	C <sub>38</sub> H <sub>72</sub> O <sub>9</sub>	672.97	H	9	9	H		
28	Rha-C <sub>10</sub> -C <sub>10</sub> -CH <sub>3</sub>	C <sub>27</sub> H <sub>50</sub> O <sub>9</sub>	518.68	H	3	3	CH <sub>3</sub>		
29	Decenoyl-Rha-C <sub>10</sub> -C <sub>10</sub>	C <sub>36</sub> H <sub>64</sub> O <sub>10</sub>	656.89	b	3	3	H		
<b>Di-rhamno-mono-lipidic congeners</b>									
30	Rha-Rha-C <sub>8</sub>	C <sub>20</sub> H <sub>36</sub> O <sub>11</sub>	452.49	H	1	-	H		
31	Rha-Rha-C <sub>10</sub>	C <sub>22</sub> H <sub>40</sub> O <sub>11</sub>	480.55	H	3	-	H		
32	Rha-Rha-C <sub>12;1</sub>	C <sub>24</sub> H <sub>42</sub> O <sub>11</sub>	506.58	H	5(-2H)	-	H		
33	Rha-Rha-C <sub>12</sub>	C <sub>24</sub> H <sub>44</sub> O <sub>11</sub>	508.60	H	5	-	H		
34	Rha-Rha-C <sub>14</sub>	C <sub>26</sub> H <sub>48</sub> O <sub>11</sub>	536.65	H	7	-	H		
<b>Di-rhamno-di-lipidic congeners</b>									
35	Rha-Rha-C <sub>8</sub> -C <sub>8</sub>	C <sub>28</sub> H <sub>50</sub> O <sub>13</sub>	594.69	H	1	1	H		
36	Rha-Rha-C <sub>8</sub> -C <sub>10</sub>	C <sub>30</sub> H <sub>54</sub> O <sub>13</sub>	622.74	H	1	3	H		
37	Rha-Rha-C <sub>10</sub> -C <sub>8</sub>	C <sub>30</sub> H <sub>54</sub> O <sub>13</sub>	622.74	H	3	1	H		
38	Rha-Rha-C <sub>10</sub> -C <sub>10;1</sub>	C <sub>32</sub> H <sub>56</sub> O <sub>13</sub>	648.78	H	3	3(-2H)	H		
39	Rha-Rha-C <sub>10</sub> -C <sub>10</sub>	C <sub>32</sub> H <sub>58</sub> O <sub>13</sub>	650.79	H	3	3	H		
40	Rha-Rha-C <sub>8</sub> -C <sub>12;1</sub>	C <sub>32</sub> H <sub>56</sub> O <sub>13</sub>	648.78	H	1	5(-2H)	H		
41	Rha-Rha-C <sub>12;1</sub> -C <sub>8</sub>	C <sub>32</sub> H <sub>56</sub> O <sub>13</sub>	648.78	H	5(-2H)	1	H		
42	Rha-Rha-C <sub>10</sub> -C <sub>12;1</sub>	C <sub>34</sub> H <sub>60</sub> O <sub>13</sub>	676.83	H	3	5(-2H)	H		
43	Rha-Rha-C <sub>12;1</sub> -C <sub>10</sub>	C <sub>34</sub> H <sub>60</sub> O <sub>13</sub>	676.83	H	5(-2H)	3	H		
44	Rha-Rha-C <sub>10</sub> -C <sub>12</sub>	C <sub>34</sub> H <sub>62</sub> O <sub>13</sub>	678.84	H	3	5	H		
45	Rha-Rha-C <sub>12</sub> -C <sub>10</sub>	C <sub>34</sub> H <sub>62</sub> O <sub>13</sub>	678.84	H	5	3	H		
46	Rha-Rha-C <sub>10</sub> -C <sub>14;1</sub>	C <sub>36</sub> H <sub>64</sub> O <sub>13</sub>	704.89	H	3	7(-2H)	H		
47	Rha-Rha-C <sub>12</sub> -C <sub>12;1</sub>	C <sub>36</sub> H <sub>64</sub> O <sub>13</sub>	704.89	H	5	5(-2H)	H		
48	Rha-Rha-C <sub>12;1</sub> -C <sub>12</sub>	C <sub>36</sub> H <sub>64</sub> O <sub>13</sub>	704.89	H	5(-2H)	5	H		
49	Rha-Rha-C <sub>12</sub> -C <sub>12</sub>	C <sub>36</sub> H <sub>66</sub> O <sub>13</sub>	706.90	H	5	5	H		
50	Rha-Rha-C <sub>12</sub> -C <sub>14</sub>	C <sub>38</sub> H <sub>70</sub> O <sub>13</sub>	734.95	H	5	7	H		
51	Rha-Rha-C <sub>14</sub> -C <sub>12</sub>	C <sub>38</sub> H <sub>70</sub> O <sub>13</sub>	734.95	H	7	5	H		
52	Rha-Rha-C <sub>14</sub> -C <sub>14</sub>	C <sub>40</sub> H <sub>74</sub> O <sub>13</sub>	763.00	H	7	7	H		
53	Rha-Rha-C <sub>14</sub> -C <sub>16</sub>	C <sub>42</sub> H <sub>78</sub> O <sub>13</sub>	791.06	H	7	9	H		
54	Rha-Rha-C <sub>16</sub> -C <sub>14</sub>	C <sub>42</sub> H <sub>78</sub> O <sub>13</sub>	791.06	H	9	7	H		
55	Rha-Rha-C <sub>16</sub> -C <sub>16</sub>	C <sub>44</sub> H <sub>82</sub> O <sub>13</sub>	819.11	H	9	9	H		
56	Rha-Rha-C <sub>14</sub> -C <sub>14</sub> -C <sub>14</sub> <sup>(d)</sup>	C <sub>54</sub> H <sub>100</sub> O <sub>15</sub>	989.36	Structure drawn separately <sup>(d)</sup>					
57	Rha-Rha-C <sub>10</sub> -C <sub>10</sub> -CH <sub>3</sub>	C <sub>33</sub> H <sub>60</sub> O <sub>13</sub>	664.82	H	3	3	CH <sub>3</sub>		
58	Decenoyl-Rha-Rha-C <sub>10</sub> -C <sub>10</sub>	C <sub>42</sub> H <sub>74</sub> O <sub>14</sub>	803.03	b	3	3	H		

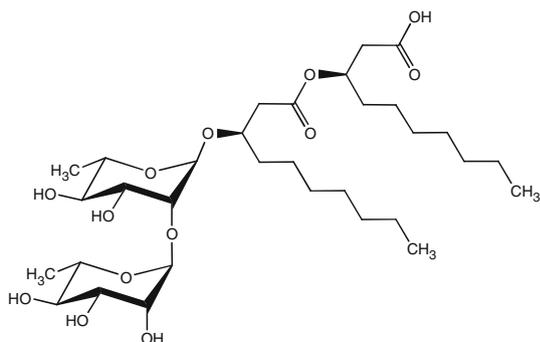
techniques, such as high-performance liquid chromatography (HPLC), mass spectrometry (MS), and HPLC/MS. Rendell et al. (1990) identified five new di-RL congeners from cultures of a clinical isolate of *P. aeruginosa*. These RLs had aliphatic chains of variable lengths and some contained an unsaturation ( $C_8-C_{10}$ ,  $C_{10}-C_8$ ,  $C_{10}-C_{12}$ ,  $C_{12}-C_{10}$ , and  $C_{10}-C_{12:1}$  as illustrated in structures **36**, **37**, **44**, **45**, and **42**, respectively, in Table 1). They also reported corresponding mono-RL congeners (structures **10**, **11**, **18**, **19**, and **16** in Table 1) using HPLC/MS. A rhamnolipid congener in which the sum of the carbons in the side chains amounted to  $C_{24}$  was identified in addition to the previous homologues by Zhang and Miller (1994) in a *P. aeruginosa* isolate; however, the exact length of each  $\beta$ -hydroxy-fatty acid was not mentioned. Déziel et al. (1999, 2000) reported, in addition to the previously identified congeners, the di-RL congeners containing  $C_8-C_8$ ,  $C_8-C_{12:1}$ ,  $C_{12:1}-C_8$ ,  $C_{12:1}-C_{10}$ ,  $C_{12:1}-C_{12}$ ,  $C_{10}-C_{14:1}$ ,  $C_{12}-C_{12}$ ,  $C_8$ ,  $C_{12:1}$ , and  $C_{12}$  moieties (structures **35**, **40**, **41**, **43**, **48**, **46**, **49**, **30**, **32**, and **33**, respectively, in Table 1), as well as other new mono-RL congeners containing  $C_8-C_8$ ,  $C_{12:1}-C_{10}$ ,  $C_8$ , and  $C_{12}$  moieties (structures **7**, **17**, **2**, and **5**, respectively, in Table 1) produced by a *P. aeruginosa* strain grown on mannitol or naphthalene as carbon sources. All in all, they reported a total number of 28 RL congeners, many of which were present in only minute concentrations.

New congeners are still regularly reported. Abalos et al. (2001) have identified new mono-RL congeners with the polyunsaturated  $\beta$ -hydroxy-fatty acid chains  $C_{8:2}$  and  $C_{12:2}$  (structures **1** and **4** in Table 1) in cultures of a *P. aeruginosa* isolate, yet their concentrations were low. Interestingly, these congeners were produced in significant amounts by a different *P. aeruginosa* isolate (AT10) in another study (Haba et al. 2003a). Additional mono-RL homologues, containing  $C_{10}-C_{14:1}$ ,  $C_{12}-C_{12:1}$ , and  $C_{10}-C_{14}$  (structures **20**, **21**, and **22** in Table 1) were detected in cultures of *Pseudomonas chlororaphis* strain NRRL B-30761 (Gunther et al. 2005). Recently, other di-RL congeners with mono-unsaturation as  $C_{10}-C_{10:1}$  and  $C_{12}-C_{12:1}$  (structures **38** and **47** in Table 1) were also identified (Sharma et al. 2007). Moreover, new mono-RLs containing  $C_8-C_{10:1}$ ,  $C_{10}-C_{10:1}$ , and  $C_{12}-C_{12}$  moieties (structures **8**, **12**, and **23** in Table 1) were identified in culture of the *P. aeruginosa* isolate LBI, although the first two congeners only represented less than 1% of all the RLs detected (Nitschke et al. 2009). Recently, other mono-RL congeners containing  $C_{14:2}$  and  $C_{10:1}-C_8$  moieties (structures **6** and **9** in Table 1) have been identified in cultures of a hyper-producing *P. aeruginosa* mutant (MIG-N146) (Guo et al. 2009).

All the previously mentioned RL congeners and homologues were identified in various isolates of *Pseudomonas* species, essentially *P. aeruginosa*. However, other RL homologues produced by non-*Pseudomonas* bacterial iso-

lates have also been reported. For example, Pajarron et al. (1993) have identified new di-RL congeners produced by a *Pseudomonas glumae* isolate, a species later renamed as *Burkholderia glumae* (Urakami et al. 1994). The major di-RL congeners they identified contained a  $C_{14}-C_{14}$  moiety; other minor congeners containing  $C_{12}-C_{14}$ ,  $C_{14}-C_{12}$ ,  $C_{14}-C_{16}$ , and  $C_{16}-C_{14}$  moieties were also identified (structures **52**, **50**, **51**, **53**, and **54** in Table 1). The major di-RL congener containing  $C_{14}-C_{14}$  was also identified in other species of *Burkholderia*, such as the pathogen *Burkholderia pseudomallei*, but as the sole RL congener (Häussler et al. 1998; Howe et al. 2006). However, Andrä et al. (2006) identified in addition to the Rha-Rha- $C_{14}-C_{14}$  congener two other RL congeners with mono- and tri-acylation with  $\beta$ -hydroxy-tetradecanoic acid: Rha-Rha- $C_{14}$  and Rha-Rha- $C_{14}-C_{14}-C_{14}$  (stated as unpublished data), respectively (structures **34** and **56** in Table 1) produced by *Burkholderia plantarii*. Apart from the work of Pajarron et al. (1993), the few above-mentioned reports on RLs produced by *Burkholderia* sp. have identified only the Rha-Rha- $C_{14}-C_{14}$  congener. However, Dubeau et al. (2009) have recently identified new mono- and di-RL homologues from *Burkholderia thailandensis* grown on glycerol or vegetable oil, with the following combinations of fatty acids:  $C_{10}-C_{12}$ ,  $C_{12}-C_{12}$ ,  $C_{12}-C_{14}$ ,  $C_{14}-C_{14}$ ,  $C_{14}-C_{16}$ , and  $C_{16}-C_{16}$ , among which, the major congeners are still those with  $C_{14}-C_{14}$  side chains. Other congeners were also found in trace amounts (Dubeau et al. 2009). Thus, the newly identified RL congeners to be added to the pool of known RLs are the mono-RLs with  $C_{12}-C_{14}$ ,  $C_{14}-C_{14}$ ,  $C_{14}-C_{16}$ , and  $C_{16}-C_{16}$  (structures **24**, **25**, **26**, and **27** in Table 1) as well as the di-RL with  $C_{16}-C_{16}$  (structures **55** in Table 1). Finally, they also identified a range of di-RL congeners produced by *B. pseudomallei* carrying the same combinations of  $C_{12}$ ,  $C_{14}$ , and  $C_{16}$  fatty acids as seen with *B. thailandensis* (Dubeau et al. 2009).

Thus, in view of the structures mentioned above, RLs can be described as follows: they are glycosides composed of rhamnose moieties (glycon part) and lipid moieties (aglycon part) linked to each other via a O-glycosidic linkage. The glycon part is composed of one (mono-RLs) or two (di-RLs) rhamnose moieties linked to each other through a  $\alpha$ -1,2-glycosidic linkage. The aglycon part, however, is mainly one or two (in few cases three)  $\beta$ -hydroxy fatty acid chains (saturated, mono-, or poly-unsaturated and of chain length varying from  $C_8$  to  $C_{16}$ ) linked to each other through an ester bond formed between the  $\beta$ -hydroxyl group of the distal (relative to the glycosidic bond) chain with the carboxyl group of the proximal chain (Fig. 1). In most cases, the carboxyl group of the distal  $\beta$ -hydroxy fatty acid chain remains free; few congeners, however, have this group esterified with a short alkyl group. Similarly, the 2-hydroxyl group of the distal (relative to the glycosidic bond) rhamnose group remains mostly free,



**Fig. 1** Chemical structure of the first identified rhamnolipid; simply named as  $\alpha$ -L-rhamnopyranosyl- $\alpha$ -L-rhamnopyranosyl- $\beta$ -hydroxydecanoate and symbolized as Rha-Rha-C<sub>10</sub>-C<sub>10</sub>. Its IUPAC names: (R)-3-[(R)-3-[2-O-( $\alpha$ -L-rhamnopyranosyl)- $\alpha$ -L-rhamnopyranosyl]oxydecanoate]oxydecanoate or the synonym name: (R)-3-[(R)-3-((2R,3R,4R,5R,6S)-4,5-dihydroxy-6-methyl-3-((2S,3R,4R,5R,6S)-3,4,5-trihydroxy-6-methyltetrahydro-2H-pyran-2-yloxy)tetrahydro-2H-pyran-2-yloxy)decanoate]oxydecanoate

although in some rare homologues it can be acylated with long chain alkenoic acid.

Variations in the chemical structures of bacterially produced RLs give rise to a large pool of RL homologues that approaches 60 structures (Table 1). The differences among these homologues come from modifications in the glycon and/or the aglycon parts, taking in consideration that variation in the aglycon part contribute largely to the biodiversity of RLs. Although few general rules apply, Déziel et al. (1999, 2000) previously noted that for RL isomers containing two fatty acid moieties of different chain lengths, the predominant (at least twice more abundant) isomeric congeners will be those with the shorter chain adjacent to the sugar. This proportion is even larger when one of the two fatty acid moieties contains a double bond, i.e., the most prevalent isomeric congener will be that with the shorter saturated fatty acid chain adjacent to the sugar while its isomeric congener with the unsaturated longer chain adjacent to the sugar is found at much lower concentrations (Déziel et al. 2000) or, in other studies, is completely absent (Sharma et al. 2007). Concerning the  $\beta$ -hydroxyl groups of the fatty acid chains, they were reported to be strictly present in the R-configuration (Bauer et al. 2006; Schenk et al. 1997).

#### Diversity of biological producers

*Pseudomonas* species have long been the main sources of RLs, with *P. aeruginosa* being considered the primary producing species. However, as noted above, many isolates from other bacterial species of varying distance in their taxonomical classification are increasingly reported to be also RL producers. First, many *Pseudomonas* species other than *P. aeruginosa* have been reported to produce RLs (Gunther et al. 2005, 2006; Onbasli and Aslim 2009). Some

belong to the same taxonomical class of *Pseudomonas* (Gammaproteobacteria) and order (Pseudomonadales) but are not part of the Pseudomonadaceae family. For instance, two *Acinetobacter calcoaceticus* isolates (order: Pseudomonadales; family: Moraxellaceae) were recently found to be RL producers (Rooney et al. 2009). Most other examples, however, are farther from *Pseudomonas* in the taxonomical class. They belong to different orders, yet under the same phylum (Gammaproteobacteria); e.g., *Pseudoxanthomonas* sp. (order: Xanthomonadales; family: Xanthomonadaceae; Nayak et al. 2009) and *Enterobacter* sp., as well as *Pantoea* sp. (order: Enterobacteriales; family: Enterobacteriaceae; Rooney et al. 2009; Vasileva-Tonkova and Gesheva 2007; Table 2).

Besides, RL-producing bacteria that do not belong to the class Gammaproteobacteria have been reported. The most prominent examples are various RL-producing *Burkholderia* spp. which are Betaproteobacteria (Andrä et al. 2006; Dubeau et al. 2009; Häussler et al. 1998; Pajarron et al. 1993). Interestingly, a *Myxococcus* sp. (class Deltaproteobacteria) has been reported to produce an unusual rhamnose-containing glycolipid (rhamnoside) called myxotyrosides which have a tyrosine-derived core structure glycosylated with rhamnose and acylated with unusual fatty acids such as (Z)-15-methyl-2-hexadecenoic and (Z)-2-hexadecenoic acid. Although these are not typical RLs, the authors claimed that the only molecules related to myxotyrosides are RLs and similarly acylated amino acids (Ohlendorf et al. 2008; Table 2).

Astonishingly, bacterial isolates belonging to different phyla than that of *Pseudomonas*, for instance, *Renibacterium salmoninarum* (Christova et al. 2004), *Cellulomonas cellulans* (Arino et al. 1998b), and *Nocardioideis* sp. (Vasileva-Tonkova and Gesheva 2005), have been reported to produce RLs, yet they belong to the phylum Actinobacteria. Another example is *Tetragenococcus koreensis*, which belongs to phylum Firmicutes and has been reported to be a RL producer (Lee et al. 2005; Table 2). The glycolipid produced by *C. cellulans* is not a typical RL. Although not fully structurally elucidated, preliminary characterizations showed it is a glycolipid whose lipid part is composed of fatty acids and hydroxy fatty acids ranging from C<sub>10</sub> to C<sub>18</sub>. The glycidic moiety is composed of glucose, rhamnose, and to a lesser extent, ribose (Arino et al. 1998b). It remains to be seen whether this glycolipid has surfactant activity.

Although bacterial species other than *P. aeruginosa* have been reported as RL producers (Table 2), it is important to note that many have not been conclusively identified (e.g., through 16S ribosomal RNA gene sequencing) nor their production of RLs have been confirmed using high-precision analytical methods (e.g., HPLC-MS). Worthy to be mentioned here, in the context of the interest in finding nonpathogenic RL-producing species for commercial production purposes, is that the pathogenicity of many of these

**Table 2** Taxonomical classification of different bacteria reported to produce rhamnolipids

Name of bacterial sp.	Culture medium	RL composition	Reference
Phylum: Actinobacteria, class: Actinobacteria			
<i>Renibacterium salmoninarum</i>	Mineral salts medium (MSM)+2% <i>n</i> -hexadecane or <i>n</i> -paraffin	Mono- and di-RL	(Christova et al. 2004)
<i>Cellulomonas cellulans</i>	Mineral salts+yeast extract+3% glycerol or 2% <i>n</i> -hexadecane	Novel rhamnose-containing glycolipid (gluco-rhamno-ribo-lipid)	(Arino et al. 1998b)
<i>Nocardioides</i> sp.	MSM+2% <i>n</i> -paraffin	RL <sup>a</sup> (congeners unidentified)	(Vasileva-Tonkova and Gesheva 2005)
Phylum: Firmicutes, class: Bacilli			
<i>Tetragenococcus koreensis</i>	Glucose yeast peptone/sodium acetate/mineral salts +2–5% (w/v) NaCl	RL (congeners unidentified)	(Lee et al. 2005)
Phylum: Proteobacteria, class: Betaproteobacteria			
<i>B. glumae</i>	Not-mentioned	Rha-Rha-C <sub>14</sub> -C <sub>14</sub> , C <sub>12</sub> -C <sub>14</sub> , and C <sub>14</sub> -C <sub>16</sub> (beside their isomers)	(Pajarron et al. 1993)
<i>B. pseudomallei</i>	Vogel–Bonner medium, glycerol medium	Di-RL congeners with C <sub>12</sub> -C <sub>12</sub> , C <sub>12</sub> -C <sub>14</sub> , C <sub>14</sub> -C <sub>14</sub> , C <sub>14</sub> -C <sub>16</sub> , and C <sub>16</sub> -C <sub>16</sub>	(Häussler et al. 1998)
<i>B. plantarii</i>	Nutrient broth (NB)+4% glycerol Mineral salts+yeast ext+soy bean oil	Rha-Rha-C <sub>14</sub> , Rha-Rha-C <sub>14</sub> -C <sub>14</sub> , and Rha-Rha-C <sub>14</sub> -C <sub>14</sub> -C <sub>14</sub>	(Andrä et al. 2006)
<i>B. thailandensis</i>	NB+4% glycerol or canola oil	Mono- and di-RL congeners with C <sub>12</sub> -C <sub>14</sub> , C <sub>14</sub> -C <sub>14</sub> , C <sub>14</sub> -C <sub>16</sub> , and C <sub>16</sub> -C <sub>16</sub>	(Dubeau et al. 2009)
Phylum: Proteobacteria, subphylum: delta/epsilon subdivision, class: Deltaproteobacteria			
<i>Myxococcus</i> sp.	Peptone medium+0.2% starch+0.2% glucose	Myxotyrosides A and B which are rhamno-amino-lipids	(Ohlendorf et al. 2008)
Phylum: Proteobacteria, class: Gammaproteobacteria			
<i>Acinetobacter calcoaceticus</i>	MSM+10% glycerol	Mono- and di-RL with C <sub>10</sub> -C <sub>10</sub>	(Rooney et al. 2009)
<i>Enterobacter asburiae</i>	MSM+10% glycerol	Mono- and di-RL with C <sub>10</sub> -C <sub>10</sub>	(Rooney et al. 2009)
<i>Enterobacter hormaechei</i>	MSM+10% glycerol	Mono- and di-RL with C <sub>10</sub> -C <sub>10</sub>	(Rooney et al. 2009)
<i>Pantoea stewartii</i>	MSM+10% glycerol	Mono- and di-RL with C <sub>10</sub> -C <sub>10</sub>	(Rooney et al. 2009)
<i>Pantoea</i> sp.	MSM+2% <i>n</i> -paraffin or kerosene	RL <sup>a</sup> (congeners unidentified)	(Vasileva-Tonkova and Gesheva 2007)
<i>P. alcaligenes</i>	Nutrient agar (NA), mineral medium+0.5% (v/v) of palm oil	Mono- and di-RL with C <sub>8</sub> -C <sub>10</sub> , C <sub>10</sub> -C <sub>10</sub> , and C <sub>10</sub> -C <sub>12</sub>	(Oliveira et al. 2009)
<i>P. aeruginosa</i>	MSM+soluble or insoluble carbon sources	Refer to text	Refer to text
<i>P. cepacia</i>	Nutrient broth	RL <sup>a</sup> (congeners unidentified)	(Onbasli and Aslim 2009)
<i>P. chlororaphis</i>	Kay's minimal medium, MSM+2% glucose, Siegmund Wagner (SW) agar	Mono-RL with C <sub>10</sub> -C <sub>8</sub> , C <sub>10</sub> -C <sub>10</sub> , C <sub>12</sub> -C <sub>10</sub> , C <sub>12:1</sub> -C <sub>10</sub> , C <sub>12</sub> -C <sub>12</sub> , C <sub>12:1</sub> -C <sub>10</sub> , C <sub>14</sub> -C <sub>10</sub> , and C <sub>14:1</sub> -C <sub>10</sub>	(Gunther et al. 2005, 2006)
<i>P. clemancea</i>	MSM+3% glycerol+high phosphate+no trace elements	Mono- and di-RL with C <sub>10</sub> -C <sub>10</sub>	(Rahman et al. 2009)
<i>P. collierea</i>	MSM+3% glycerol+high phosphate+no trace elements	Mono- and di-RL with C <sub>10</sub> -C <sub>10</sub>	(Rahman et al. 2009)
<i>P. fluorescens</i>	Bushnell–Haas Broth+0.1 mg yeast, 0.1 X NB+2,000 IU penicillin	RL <sup>a</sup> (congeners unidentified)	(Husain 2008)
<i>P. fluorescens</i>	NA, NB, MSM+2% (v/v) olive oil and 1 g/L ammonium nitrate	RL <sup>a</sup> (congeners unidentified)	(Abouseoud et al. 2008a, b)
<i>P. fluorescens</i>	NB, tryptic soy broth (TSB), minimal medium+petrol 0.1% v/v	RL <sup>a</sup> (congeners unidentified)	(Wilson and Bradley 1996)
<i>P. fluorescens</i>	MSM+2% <i>n</i> -paraffin or kerosene	RL <sup>a</sup> (congeners unidentified)	(Vasileva-Tonkova et al. 2006)

**Table 2** (continued)

Name of bacterial sp.	Culture medium	RL composition	Reference
<i>P. fluorescens</i>	Nutrient broth	RL <sup>a</sup> (congeners unidentified)	(Onbasli and Aslim 2009)
<i>P. luteola</i>	Sugar beet molasses at 5% w/v	RL <sup>a</sup> (congeners unidentified)	(Onbasli and Aslim 2009)
<i>P. putida</i>	Mineral salts+yeast extract+glucose+corn oil	RL, the exact structures were not determined	(Martinez-Toledo et al. 2006)
<i>P. putida</i>	Sugar beet molasses at 5% w/v	RL <sup>a</sup> (congeners unidentified)	(Onbasli and Aslim 2009)
<i>P. putida</i>	Mineral salt agar+2% hexadecane	RL <sup>a</sup> (congeners unidentified)	(Tuleva et al. 2002)
<i>P. putida</i>	Mineral salts medium+phenanthrene	Suggested to be RL <sup>a</sup>	(Cuny et al. 2004)
<i>P. stutzeri</i>	MSM+crude oil 1%	RL <sup>a</sup> (congeners unidentified)	(Celik et al. 2008)
<i>P. stutzeri</i>	MSM+glucose 10%	RL <sup>a</sup> (congeners unidentified)	(Janiyani et al. 1992)
<i>P. stutzeri</i>	Nutrient broth	RL <sup>a</sup> (congeners unidentified)	(Onbasli and Aslim 2009)
<i>P. teessidea</i>	MSM+3% glycerol+high phosphate+no trace elements	Mono- and di-RL with C <sub>10</sub> -C <sub>10</sub>	(Rahman et al. 2009)
<i>Pseudoxanthomonas</i> sp.	MSM+2% mannitol	Not definitely confirmed	(Nayak et al. 2009)

<sup>a</sup> Identity of RL has not been definitively confirmed

rare, newly discovered RL-producers is often uncertain and will require further investigations. These novel isolates, reported to be RL producers, have been found distributed within and outside the class Gammaproteobacteria. This highlights the need for wide spectrum screening programs for RL producers that encompass different orders of Gammaproteobacteria or even of other classes. Still, some authors anticipate that novel RL-producing isolates will be found mostly within the class Gammaproteobacteria (Rooney et al. 2009). Finding new RL-producing bacteria is beneficial from a biotechnological point of view as it might result into the discovery of producers that are nonpathogenic compared to the pathogenic *P. aeruginosa* strains and hence become more appropriate candidates for the industrially-safe production of RLs.

#### Genetic basis of diversity of composition of natural RL mixtures

Naturally produced RL biosurfactants are always found as mixtures of different RL congeners, as observed with the various strains of *P. aeruginosa* (Abalos et al. 2001; Abdel-Mawgoud et al. 2009; Benincasa et al. 2004; Haba et al. 2003a; Mata-Sandoval et al. 1999; Pornsunthorntaweew et al. 2008), of *P. chlororaphis* (Gunther et al. 2005, 2006) and of different *Burkholderia* spp. such as *B. glumae* (Pajarron et al. 1993), *B. plantarii* (Andrä et al. 2006), *B. thailandensis*, and *B. pseudomallei* (Dubeau et al. 2009). Variations in the nature and distribution of the different RL congeners identified in various RL mixtures might be attributed to diverse cultivation conditions as well as to strain-related variations (Déziel et al. 1999, 2000; Dubeau et al. 2009). These mixtures of congeners display physicochemical properties which together would probably be very different from the individual RL congeners.

On the genetic level, biosynthesis of RLs in *P. aeruginosa* occurs through three sequential steps (Soberón-Chávez et al. 2005): (1) RhIA (encoded by *rhlA* gene) is involved in the synthesis of the fatty acid dimer (HAAs) moiety of RLs from 3-hydroxyfatty acid precursors (Déziel et al. 2003; Lépine et al. 2002; Zhu and Rock 2008); (2) the membrane-bound RhIB rhamnosyltransferase (encoded by *rhlB* gene) uses dTDP-L-rhamnose and a HAA molecule as precursors, yielding mono-RL (Ochsner et al. 1994); and (3) these mono-RLs are in turn the substrates, together with dTDP-L-rhamnose, of the RhIC rhamnosyltransferase (encoded by *rhlC* gene) to produce di-RLs (Rahim et al. 2001). In *P. aeruginosa*, a bicistronic *rhlAB* operon encodes the first two enzymes while *rhlC* is elsewhere on the genome. While *P. aeruginosa* produces a mixture of mono- and di-RLs, *P. chlororaphis* has been reported to produce only mono-RLs. It was suggested that this species lack a homologue of the *rhlC* gene which encodes for the second rhamnosyltransferase responsible for biosynthesis of di-RLs (Gunther et al. 2005).

The genes responsible for RL synthesis in *B. thailandensis* and *B. pseudomallei* were recently reported (Dubeau et al. 2009). These bacterial species harbor two paralogous *rhl* gene clusters carrying homologues of *rhlA*, *rhlB*, and *rhlC*. However, unlike in *P. aeruginosa*, these three genes are grouped together in the same gene cluster. Using *rhlA1*, *rhlA2*, and *rhlA1rhlA2* mutants, both *rhl* gene cluster paralogues were shown to contribute to the total RL production of *B. thailandensis* (Dubeau et al. 2009). While until then only di-RL congeners had been found to be produced by *Burkholderia* species, Dubeau et al. demonstrated the co-production of mono-RL congeners together with the di-RLs by *B. thailandensis*, although the former congeners were produced at in minimal concentrations (Dubeau et al. 2009). The ratio of di-RLs to mono-RLs

found in *B. thailandensis* is approximately 13:1, whereas that in *P. aeruginosa* is only 4:1 (Dubeau et al. 2009). It is hypothesized that since *P. aeruginosa* harbors *rhIA* and *rhIB* in one operon and *rhIC* in another, this may result in different levels and timing of expression of the first and second rhamnosyltransferases (RhIB and RhIC, respectively), thus decoupling both activities. On the other hand, *Burkholderia* species co-express *rhIB* and *rhIC*, which might favor the simultaneous production of all the enzymes of the biosynthetic pathway in the same stoichiometric ratio. In other words, RhIC will be produced at the same time and in an amount equivalent to that of RhIB and thus would efficiently add another rhamnosyl group on most of the mono-RLs produced by RhIB.

#### Diversity of physiological functions and roles

Although numerous studies have been performed in the field of RLs, the exact physiological functions of these molecules for the producing bacteria are still not precisely defined. Many of these functions are derived from the well-known physicochemical properties of RLs such as surface activity, wetting ability, detergency, and other amphipathic-related properties. Taken together, studies suggest that these molecules might play multifunctional roles for the producing organisms.

#### *RLs promote the uptake and biodegradation of poorly soluble substrates*

Most bacteria degrading hydrophobic substrates release biosurfactants that facilitate the uptake and assimilation of hydrocarbons (Hommel 1994). For instance, linear alkanes are very insoluble in water, their solubility decrease as the molecular weight increases, but they are very good nutrient sources for *P. aeruginosa*. With the exception of *n*-alkanes of very low molecular weight, solubility values of these molecules are well below the micromolar range (e.g.,  $2 \times 10^{-10}$ M for hexadecane). This impedes their sufficient uptake by bacteria, and it is thus not obvious how these hydrophobic molecules enter the cells to sustain growth. For medium- and long-chain-length *n*-alkanes, it is likely that bacteria gain access to them either by a surfactant-facilitated process (emulsification and solubilisation) or by directly adhering to hydrocarbon droplets (surfactant-mediated increase of cell surface hydrophobicity).

Because of their surface tension-reducing and emulsifying activities, RLs have been considered as surfactants. As a result, these amphiphilic molecules have been mostly studied for their ability to mediate the assimilation of hydrophobic substrates in liquid cultures, especially hydrocarbons such as *n*-alkanes (Beal and Betts 2000; Hisatsuka et al. 1971; Itoh and Suzuki 1972; Koch et al. 1991;

Noordman and Janssen 2002; Shreve et al. 1995). Interestingly, RLs also enhance the biodegradation of poorly soluble molecules by causing the cell surface to become more hydrophobic (Al-Tahhan et al. 2000; Shreve et al. 1995; Zhang and Miller 1994). The few investigations performed with RL-nonproducing mutants are especially revealing: Beal and Betts (2000), using an *rhIA* mutant, have shown that endogenous production of RLs indeed enhances the biodegradation of hexadecane by *P. aeruginosa*, but that their contribution is relatively limited. In support of the notion that the specific function of RLs is unlikely to be solely to facilitate the assimilation of insoluble substrates, these exoproducts are as efficiently synthesized when the cells are grown on soluble substrates such as carbohydrates (Guerra-Santos et al. 1984).

#### *RLs as immune modulators and virulence factors*

The airway mucosa is an efficient barrier to protect the host from infections. Nevertheless, presence of noninvasive microorganisms in the airways can lead to damage to the respiratory mucosa due to the production of extracellular toxins. For instance, *P. aeruginosa* causes serious infections in immunocompromised patients and individuals suffering from cystic fibrosis (CF). Probably because they do not present the profile of typical or traditional virulence factors, RLs are generally not considered significant members of the arsenal of *P. aeruginosa*. However, a resurgence in recent literature is bringing back to the forefront some older investigations, which, together, strongly demonstrate their relevance as virulence determinants.

Kownatzki et al. (1987) found that sputum samples obtained from *P. aeruginosa*-colonized CF patients contained RLs (up to 8  $\mu$ g/ml) and reported a correlation between elevated levels of RLs and worsened patient's clinical status. These RL concentrations in sputum samples, however, might actually underestimate the concentrations present in the lower respiratory tract, as levels of 65  $\mu$ g/ml were found in secretions of a lung removed from a CF patient (Read et al. 1992).

*P. aeruginosa* had been known for a long time to produce a solvent-soluble glycolipid with hemolytic activity when, a few years after RLs were reported, the coincidence between the two was noticed (Sierra 1960). Nevertheless, the heat-stable extracellular hemolysin designation for RLs has persisted for many years (Fujita et al. 1988; Johnson and Boese-Marrazzo 1980; Kurioka and Liu 1967; Stutts et al. 1986). The di-RL Rha-Rha-C<sub>14</sub>-C<sub>14</sub> from *B. pseudomallei* is similarly hemolytic for various erythrocyte species and also cytotoxic at high concentrations for nonphagocytic and phagocytic cell lines (Häussler et al. 1998).

Actually, purified RLs act directly on immune cells. Among many exoproducts released by *P. aeruginosa* that affects neutrophil recruitment, RLs have been shown to

induce direct neutrophil chemotactic activity (Kharazmi et al. 1989). Preincubation of monocytes with RLs enhanced the oxidative burst response of these cells (Kharazmi et al. 1989). RLs, especially di-RLs, are cytolytic for human monocyte-derived macrophages (McClure and Schiller 1992), and at lower concentrations, they can also inhibit the phagocytic response of macrophages (McClure and Schiller 1996). Tissue-cultured macrophages treated with the *B. pseudomallei* di-RLs internalized fewer viable *B. pseudomallei* bacteria (Häussler et al. 2003). It is worth mentioning here that RLs are known to induce lysis of the amoebae *Dictyostelium discoideum* (Cosson et al. 2002). Rha-Rha-C<sub>14</sub>-C<sub>14</sub> is also produced by *B. plantarii* and was found to be endowed with endotoxin-like properties (Andrä et al. 2006). *P. aeruginosa* RLs induce histamine release from mast cells (Bergmann et al. 1989) and stimulate the generation and release of the inflammatory mediators serotonin and 12-hydroxyeicosatetraenoic acid from human platelets (Konig et al. 1992). RLs stimulate the copious release of interleukin (IL)-8, granulocyte-macrophage colony-stimulating factor, and IL-6 from nasal epithelial cells at non-cytotoxic levels (Bédard et al. 1993). RLs induce the lysis of polymorphonuclear leukocytes (PMNs; Jensen et al. 2007; Shryock et al. 1984; Van Gennip et al. 2009) while, at subtoxic levels, they only stimulate both chemotaxis and chemokinesis of PMNs (Shryock et al. 1984). Furthermore, Alhede et al. (2009) recently demonstrated that exposure to PMNs triggers the production of RLs by biofilm-growing *P. aeruginosa* cells, whereas in vitro, their biofilms usually produce very little RLs (Alhede et al. 2009; Morici et al. 2007), suggesting that biofilm-associated RLs function as a shield against PMNs. These studies illustrate that RLs probably contribute to the inflammatory-related tissue damage observed in lungs of CF patient.

RLs inhibit ciliary function and produce damage to the bronchial epithelium: they alter respiratory epithelial ion transport through reduced sodium absorption and unidirectional chloride fluxes across human bronchial epithelium (Stutts et al. 1986), inhibit transcellular ion transport, as observed by a decrease in amiloride-sensitive short-circuit current across sheep tracheal epithelium (Graham et al. 1993), interfere with the normal tracheal ciliary function, as shown on ciliary function in rabbit tracheal epithelium (Hingley et al. 1986), and confirmed by a slowing of ciliary beat frequency of human airway epithelium in vitro (Kanthakumar et al. 1996; Read et al. 1992). They also stimulate the release of mucus glycoconjugates from feline trachea or human bronchial mucosa (Fung et al. 1995; Somerville et al. 1992). More recently, Zulianello et al. (2006) have shown that *P. aeruginosa* requires the production of RLs to invade respiratory epithelia reconstituted with primary human respiratory cells.

Finally, the importance of RL production for establishment of an actual infection was recently directly shown with two in vivo models: compared to the wild type strain, an *rhlA* mutant was more rapidly cleared from both mice models of intraperitoneal foreign-body infection and pulmonary infection (Van Gennip et al. 2009).

#### *RLs as antimicrobials*

Early on, the wide-ranging antimicrobial properties of RLs were noted (Itoh et al. 1971). Especially, they were shown to be active against a large variety of bacteria, including both Gram-negative and Gram-positive species, though the latter, such as *Bacillus subtilis*, are in general much more susceptible to the toxic effect of RLs (Itoh et al. 1971; Lang et al. 1989). Growth inhibition was also observed against *Rhodococcus erythropolis* and *Bacillus cereus* (Arino et al. 1998a).

The target of synthetic surfactants is the cell envelope. Likewise, the proposed mechanism of action of RLs consists of intercalation into the biological membrane and destruction by their permeabilizing effect (Sotirova et al. 2008).

The group of Manresa has performed comprehensive investigations on the antimicrobial properties of mixtures of RL congeners produced by three different strains of *P. aeruginosa* grown on various types of vegetable oil wastes (Abalos et al. 2001; Benincasa et al. 2004; Haba et al. 2003b). The various RL combinations displayed antimicrobial activity against nearly all tested Gram-positives species, including *Staphylococcus*, *Mycobacterium*, and *Bacillus*, and significant activity against a number of Gram-negative species, with *Serratia marcescens*, *Enterobacter aerogenes*, and *Klebsiella pneumoniae* as the especially sensitive ones. They also found an excellent inhibitory activity against a range of fungal species, including the filamentous fungi *Chaetomium globosum*, *Aureobacidium pullulans*, and *Gliocladium virens*, and the phytopathogens *Botrytis cinerea* and *Rhizoctonia solanii*, but no significant effect on yeasts (Abalos et al. 2001; Benincasa et al. 2004; Haba et al. 2003b).

RLs were shown to exert high zoosporicidal activity, probably through zoospore lysis, against various zoosporic phytopathogens, including species from the *Pythium*, *Phytophthora*, and *Plasmopara* genera (Stanghellini and Miller 1997). Interestingly, fluorescent pseudomonads are effective in the biological control of plant pathogens. Recently, the biocontrol activity of *P. aeruginosa* strain PNA1 was reported to require the production of RLs, as an *rhlA* mutant was much less successful at inhibiting plant disease caused by *Pythium* sp. (Perneel et al. 2008).

Furthermore, antiviral, algicidal, mycoplasmicidal, and anti-amoebal properties of RLs have also been reported (Cosson et al. 2002; Haferburg et al. 1987; Itoh et al. 1971; Wang et al. 2005).

### RLs in surface motility

Unlike swimming motility, which bacteria use to move individually in the aqueous phase, swarming motility is a form of rapid and coordinated translocation of a bacterial population across a surface, often resulting in flowery, dendritic colonies (Fraser and Hughes 1999; Verstraeten et al. 2008). Swarmer cells move along their long axis in multicellular rafts, are often hyperflagellated and elongated, and are surrounded by a self-produced “slime” mainly composed of exopolysaccharides and surface-active compounds (Harshey 2003). A key feature of swarming cells is surface conditioning achieved by the production of a wetting agent that reduces the surface tension surrounding the colony (Matsuyama and Nakagawa 1996).

In *P. aeruginosa*, swarming motility was relatively recently reported (Déziel et al. 2001; Köhler et al. 2000; Rashid and Kornberg 2000) and shown to require flagella and the production of a wetting agent, at least HAAs, and ideally also RLs (Caiazza et al. 2005; Déziel et al. 2003; Köhler et al. 2000). A notable exception was noted by ShROUT et al. (2006) who were able to see swarming of an *rhlAB* mutant. Recently, Tremblay et al. (2007) reported that HAAs and di-RLs actually modulate the development of the fractal-like patterns formed by migrating *P. aeruginosa* swarming colonies. They found that di-RLs promote tendrill formation and migration, acting like self-produced chemotactic attractants, while HAAs play the opposite role, repelling swarming tendrills. Mono-RLs seem to act solely as wetting agent. These findings extended the previous work by Caiazza et al. (2005) who identified a role for RLs in maintenance of the swarming colony organization.

An *rhlA* mutant was also reported to display reduced twitching motility, a form of surface translocation requiring type IV pili but not flagella (Pamp and Tolker-Nielsen 2007). Finally, under the exact same conditions of swarming, *P. aeruginosa* expressing neither flagella nor type IV pili displays another type of motility called sliding, which was also shown to be promoted by the production of RLs (Murray and Kazmierczak 2008).

### RLs in biofilm development

More and more studies are finding that certain factors which affect swarming motility also influence other bacterial multicellular behaviors, especially biofilm formation (Harshey 2003; Verstraeten et al. 2008). *P. aeruginosa* mutants with altered swarming are defective in “normal,” mature biofilm formation (Overhage et al. 2007; ShROUT et al. 2006; Yeung et al. 2009). Indeed, mutants displaying enhanced swarming are much less prone to form a biofilm (Caiazza et al. 2007; Parkins et al. 2001), presumably because the cells do not efficiently settle on the substratum.

Accumulating data therefore support the model that swarming motility is required as a critical step in biofilm development.

One explanation for the fundamental link between swarming motility and biofilms in *P. aeruginosa* is being revealed: RLs are also involved in biofilm development. First, exogenous RLs induce a release of lipopolysaccharides and thus enhance cell surface hydrophobicity, which might favor primary adhesion of planktonic cells (Al-Tahhan et al. 2000; Zhang and Miller 1994). RL production appears as a means for regulation of cell-surface hydrophobicity and modification of adhesive interactions, especially when changes in nutritional conditions occur (Boles et al. 2005; de Andrés et al. 1991; Déziel et al. 2003; Neu 1996).

RLs facilitate the surface-associated migration of bacteria in the biofilm and therefore the initial microcolony formation and differentiation of the biofilm structure (Pamp and Tolker-Nielsen 2007). Using fluorescent reporter genes, Lequette and Greenberg (2005) have shown that the expression of *rhlAB* is modulated by the localization of cells in the biofilm. Actually, a critical role for RLs seems to involve interference with both cell–cell and cell–substratum interactions, as they were shown to mediate the detachment and dispersion of *P. aeruginosa* cells from the biofilm, especially from the center of microcolonies (Boles et al. 2005; Schooling et al. 2004) and to maintain open the channels inside the biofilm during late stages of biofilm development (Davey et al. 2003). Accordingly, overproduction of RLs by *P. aeruginosa*, as observed for instance in *algR* mutant biofilms (Morici et al. 2007), disrupts the structure or impede biofilm formation (Davey et al. 2003). Exogenous RLs interfere with normal biofilm infrastructure, and this detachment effect seems nonspecific, as addition of RLs disrupts *Salmonella typhimurium* and *Bordetella bronchiseptica* biofilms as well (Irie et al. 2005; Mireles et al. 2001).

### Perspectives

In this mini-review, we have presented a detailed assessment of the diversity of RL structures, RL-producing microorganisms, and physiological functions of RLs for these producing bacteria. Clearly, the widening variety of microbes identified as RL producers will entice researchers to screen for nonpathogenic and industrially safe alternatives to *P. aeruginosa*, the well-known opportunistic human pathogen. However, it must be emphasized that in most cases when a new bacterial species was reported to produce RLs, often only one isolate was actually selected for further studies. Therefore, for now, only few bacterial species can be considered actual RL producers. Screening efforts will hence require the development of wide spectrum approaches, probably based on the detection of surface tension lowering substance or, theoretically and ideally, the presence of the *rhlA*, *rhlB*, and *rhlC* homologues in the genomes of bacteria.

Likely, there will be trials to separate the main RL homologues in pure forms to explore their individual biological and surface activities for a variety of applications. This approach may unravel the promising biotechnological uses of these glycolipid biosurfactants either in pure form or in mixtures. Moreover, characterization of the structure-activity relationship of these compounds might be useful for prediction of the behavior of different natural RL mixtures comprising one major congener among other minor congeners.

Finally, it is expected that studies on the genetic regulation of RL synthesis, especially in the context of specific environmental conditions affecting the requirement for the production of these biosurfactants, will help in deciphering the exact genetic control of RL production. Consequently, this understanding should lead to more successful control of regulatory and growth factors directing the optimized large-scale production with a safe host.

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