Inhibition of swarming and virulence factor expression in *Proteus mirabilis* by resveratrol

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Resveratrol (3,5,4-trihydroxy-trans-stilbene) is a phytoalexin compound with anti-inflammatory and antioxidant activities. The effect of resveratrol on swarming and virulence factor expression of *Proteus mirabilis*, an important pathogen infecting the urinary tract, was determined on swarming agar plates with and without the compound. Bacteria harvested at different times were assayed for cell length and the production of flagella, hemolysin and urease. Resveratrol inhibited *P. mirabilis* swarming and virulence factor expression in a dose-dependent manner. Resveratrol significantly inhibited swarming at 15 μg ml⁻¹, and completely inhibited swarming at 60 μg ml⁻¹. Inhibition of swarming and virulence factor expression was mediated through RsbA, a His-containing phosphotransmitter of the bacterial two-component signalling system possibly involved in quorum sensing. Complementation of an *rsbA*-defective mutant with the *rsbA* gene restored its responsiveness to resveratrol. The compound also inhibited the ability of *P. mirabilis* to invade human urothelial cells. These findings suggest that resveratrol has potential to be developed as an antimicrobial agent against *P. mirabilis* infection.

INTRODUCTION

*Proteus mirabilis*, a motile Gram-negative enteric bacterium, is an important pathogen of the urinary tract, and is the primary infectious agent in patients with indwelling urinary catheters (Warren et al., 1982). Individuals suffering from urinary tract infections caused by *P. mirabilis* often develop bacteriuria, cystitis, kidney and bladder stones, catheter obstruction due to stone encrustation, acute pyelonephritis, and fever (Burall et al., 2004; Johnson et al., 1993; Mobley & Warren, 1987).

The ability of *P. mirabilis* to colonize the surfaces of catheters and the urinary tract is believed to be aided by a characteristic known as swarming differentiation and migration (Allison et al., 1994). *P. mirabilis* swarming involves the coordinate differentiation of short, motile, vegetative cells with a few peritrichous flagella into multinucleate, aseptate swarm cells of up to 40 times the vegetative cell length, and with more than a 50-fold greater surface density of flagella. The swarm cells migrate coordinately and rapidly away from the colony as multicellular rafts until they pause (consolidation) and undergo some de-differentiation (Allison & Hughes, 1991b; Rauprich et al., 1996). Regular cycles of migration and consolidation generate a colony on the agar surface with a characteristic pattern of concentric rings (Allison & Hughes, 1991b; Rauprich et al., 1996).

Several potential virulence factors may be responsible for the pathogenicity of *P. mirabilis*. Among them, flagella, necessary for motility, are involved in establishing infection (Harmon et al., 1989). Urease, which is responsible for the formation of bladder and kidney stones at later stages of infection (Mobley & Hausinger, 1989), can facilitate the colonization of the urinary tract in a mouse model (Jones et al., 1990). Haemolysin, which is cytotoxic for cultured urinary tract epithelial cells (Mobley et al., 1991), has been shown to be correlated with the ability of bacteria to invade cells (Peerbooms et al., 1984). The ability of *P. mirabilis* to express virulence factors, including urease and haemolysin, and to invade human urothelial cells, is coordinately regulated with swarming differentiation (Allison & Hughes, 1991a; Allison et al., 1992b; Liaw et al., 2000, 2001, 2004). Characterization of *Proteus* mutants has indicated that a substantial number of proteins, including FlhD-C2, FlhA, Umo, Lrp, RsbA, RsmA, SpeB and others, are involved in regulation of swarming and virulence factor expression.
expression (Dufour et al., 1998; Fraser & Hughes, 1999; Gygi et al., 1995a; Hay et al., 1997). Among these regulatory proteins, RsbA, which has been suggested to be a His-containing phosphotransmitter of the bacterial two-component signalling system (Belas et al., 1998; Liaw et al., 2001; Takeda et al., 2001), has been shown by us to act as a negative regulator of swarming differentiation and virulence factor expression in P. mirabilis (Liaw et al., 2001, 2004).

Resveratrol (3,5,4-trihydroxy-trans-stilbene) is a naturally occurring phytoalexin, one of a group of compounds that are produced in plants during the stress of attack by pathogens (Dong, 2003; Jeandet et al., 2002). Invasion of grapevines by fungi induces the production of resveratrol to ward off the damaging microbes (Jeandet et al., 2002). The compound is found in some foods and drinks, including red wine, grapes and peanuts. Resveratrol has been shown to have anti-inflammatory, anti-proliferative, antiviral and antioxidant activities (Jeandet et al., 2002). Intensive research has been directed to the role of resveratrol in human health, primarily because of its protective effects against cardiac ailments and cancer (Jeandet et al., 2002). Recent studies have indicated that resveratrol has growth-inhibitory effects on some bacterial pathogens (Chan, 2002; Docherty et al., 2001; Mahady & Pendland, 2000; Tegos et al., 2002). In the course of studying the effect of resveratrol on human pathogens, we found that resveratrol could inhibit swarming and virulence factor expression in P. mirabilis. This finding suggests that resveratrol has the potential to be developed as an antimicrobial agent against P. mirabilis.

RESULTS

Inhibition of P. mirabilis swarming by resveratrol

To test the effect of resveratrol on P. mirabilis swarming, the bacteria were inoculated onto the centre of LB swarming agar plates containing various concentrations of resveratrol, and the migration distance of the bacteria was measured. Resveratrol significantly inhibited swarming migration of P. mirabilis at concentrations as low as 15 μg ml⁻¹ (Fig. 1b, lower panel); swarming was inhibited in a dose-dependent manner. At a concentration of 60 μg ml⁻¹, resveratrol completely blocked the swarming ability of P. mirabilis (Fig. 1a, b). Previously, we have reported that rsbA-defective P. mirabilis mutants have a higher ability to swarm and to express virulence factors, and have suggested that RsbA acts as a repressor of swarming and virulence factor expression (Liaw et al., 2001). To investigate whether RsbA plays a role in resveratrol-induced swarming inhibition, an rsbA-defective mutant, P1100, was tested for its response to resveratrol. Consistent with our previous report (Liaw et al., 2001), the rsbA-defective mutant migrated further in a given time than the wild-type cells. Whereas swarming of wild-type P. mirabilis was inhibited by resveratrol, that of the rsbA-defective mutant was not (Fig. 1a, b). These data indicate that resveratrol inhibited P. mirabilis swarming through an RsbA-dependent pathway. The inhibitory effect of resveratrol on swarming might arise from a toxic effect on the bacteria. We therefore tested whether resveratrol affected the growth rate of P. mirabilis. As shown in Fig. 1(c), the growth rate of both wild-type and rsbA-defective P. mirabilis was inhibited slightly by resveratrol at 30 and 60 μg ml⁻¹. At 16 h post-inoculation, the bacteria grew to similar densities, regardless of the presence of resveratrol. Considering the fact that resveratrol could inhibit swarming but only marginally inhibit growth in wild-type P. mirabilis, and that resveratrol slightly inhibited growth but not swarming in mutant P. mirabilis, we concluded that the inhibitory effect of resveratrol on swarming was unlikely to be due to inhibition of cell growth.

Inhibition of swarming differentiation of P. mirabilis by resveratrol

Knowing that the swarming behaviour of P. mirabilis was inhibited by resveratrol, experiments were performed to determine whether swarming differentiation of P. mirabilis was also affected by resveratrol. Wild-type and rsbA-defective mutant cells were spread on LB swarming agar plates in the presence or absence of resveratrol. Cell length, a marker of cell differentiation, was measured 2 h after seeding and hourly thereafter. As shown in Fig. 2(a), in the absence of resveratrol, the rsbA-defective mutant formed longer cells than the wild-type strain during the 7 h incubation period, and became longest about 1 h earlier than wild-type cells. Moreover, the rsbA-defective mutant retained its elongated cell shape for longer than the wild-type strain. These data, consistent with our previous report

METHODS

Chemicals. All chemicals were purchased from Sigma.

Bacterial strains and growth conditions. The bacterial strains used in this study were the wild-type P. mirabilis P19, a P. mirabilis P19 rsbA-defective mutant (P1100), and a RsbA-complemented strain (Pc) (Liaw et al., 2001, 2004). Bacteria were routinely cultured at 37 °C in Luria–Bertani (LB) medium.

Swarming behaviour assay. The swarming migration distance assay was performed as described previously (Gygi et al., 1999b; Liaw et al., 2001). Briefly, an overnight bacterial culture (5 μl) was inoculated centrally onto the surface of dry LB swarming agar (2%, w/v) plates with or without resveratrol, which were then incubated at 37 °C. The swarming migration distance was assayed by following swarm fronts of the bacterial cells and recording progress at 30 or 60 min intervals.

Measurement of cell length, haemolysin and urease activities, and flagellin level. Cell length, cell membrane-associated haemolysin activity, urease activity in whole-cell suspensions, and flagellin, were assayed as described previously (Liaw et al., 2001, 2004).

Cell invasion assay. Wild-type or rsbA-defective P. mirabilis grown overnight in the presence or absence of resveratrol (30 μg ml⁻¹) were used to infect a human urothelial NTUB1 cell line, which was originally derived from a urinary bladder carcinoma, and was obtained from the National Taiwan University Hospital. The cell-invasion ability of the bacteria was then assayed as described previously (Liaw et al., 2000, 2001).
(Liaw et al., 2001), indicate that the rsbA-defective mutant differentiated earlier and maintained a differentiated state for longer than the wild-type strain.

When resveratrol was added to the LB swarming agar plates, the length of the wild-type cells was reduced, but that of the rsbA-defective mutant cells was not (Fig. 2a, b). These data

**Fig. 1.** (a) Effect of resveratrol on the swarming of wild-type and rsbA-defective *P. mirabilis*. The histogram shows the swarming migration distance of wild-type and rsbA-defective *P. mirabilis* in the presence of various concentrations of resveratrol (0, 30 or 60 µg ml\(^{-1}\)). The data represent the mean and SD of four independent experiments. w, wild-type; m, rsbA-defective mutant. (b) Halo images of swarming plates containing different concentrations of resveratrol (0, 15, 30 or 60 µg ml\(^{-1}\)) at 8 h after inoculation. (c) Effect of resveratrol on the growth of wild-type and rsbA-defective *P. mirabilis*. Overnight bacterial culture was diluted 1:100 to fresh LB broth containing different concentrations of resveratrol: 0 µg ml\(^{-1}\) (●), 30 µg ml\(^{-1}\) (■) or 60 µg ml\(^{-1}\) (△). The bacterial growth was monitored thereafter as OD\(_{600}\). Data are the mean of three determinations.
indicate that resveratrol inhibited swarming differentiation of *P. mirabilis* and that this inhibition was mediated through an RsbA-dependent pathway.

This conclusion was also confirmed by the finding that resveratrol could inhibit flagellin production in wild-type, but not in mutant, *P. mirabilis* (Fig. 2c).

**Inhibition of virulence factor expression in *P. mirabilis* by resveratrol**

In *P. mirabilis*, expression of virulence factors is regulated by RsbA coordinately with swarming differentiation (Liaw *et al.*, 2001). We next tested whether expression of virulence factors, including haemolysin and urease, was also affected.
by resveratrol in *P. mirabilis*. As shown in Fig. 3, in the absence of resveratrol, the *rsbA*-defective mutant expressed higher levels of haemolysin and urease activity than the wild-type strain during the 7 h incubation period, consistent with our previous report (Liaw *et al.*, 2001). Resveratrol inhibited the haemolysin and urease activity of wild-type *P. mirabilis*, but not that of the *rsbA*-defective mutant, indicating that resveratrol could inhibit the expression of haemolysin and urease in *P. mirabilis* through an RsbA-dependent pathway.

### Inhibition of the cell-invasion ability of *P. mirabilis* by resveratrol

Swarming differentiation and expression of virulence factors are correlated with the ability of *P. mirabilis* to invade cells (Allison *et al.*, 1992a, b). Knowing that swarming differentiation and virulence factor expression were inhibited by resveratrol, we next tested whether resveratrol could inhibit the cell-invasion ability of wild-type and *rsbA*-defective *P. mirabilis*. As shown in Table 1, the *rsbA*-defective mutant exhibited higher cell-invasion ability than wild-type cells, consistent with our previous report (Liaw *et al.*, 2001). Resveratrol inhibited the cell-invasion ability of wild-type *P. mirabilis*, but not that of the *rsbA*-defective mutant, indicating that resveratrol could inhibit the cell-invasion ability of *P. mirabilis* through an RsbA-dependent pathway.

#### Complementation of the *rsbA*-defective mutant by wild-type *rsbA* restores wild-type responsiveness to resveratrol

To further confirm that loss of responsiveness to resveratrol in the *rsbA*-defective mutant is indeed due to the defective *rsbA* gene, an *rsbA*-complemented strain (Pc), which was generated by transforming the wild-type *rsbA* gene into the *rsbA*-defective mutant (Liaw *et al.*, 2001), was tested for its responsiveness to resveratrol. As shown in Fig. 4(a), in the absence of resveratrol, while the *rsbA*-defective mutant exhibited the superswarming phenotype, the *rsbA*-complemented strain exhibited swarming behaviour similar to that of wild-type *P. mirabilis*. In the presence of resveratrol, swarming of the *rsbA*-defective mutant was not inhibited, but that of the *rsbA*-complemented strain was inhibited to a level similar to that of the wild-type strain. Together, these data indicate that expression of RsbA in the *rsbA*-defective mutant led to the restoration of responsiveness to resveratrol, and suggest that inhibition of swarming by resveratrol is mediated through RsbA and not through other proteins in *P. mirabilis*. We also tested whether resveratrol could inhibit haemolysin expression in the *rsbA*-complemented strain as it could in the wild-type strain. As shown in Fig. 4(b), while the haemolysin activity of the *rsbA*-defective mutant was not inhibited by resveratrol, the activity of the *rsbA*-complemented strain was inhibited to a level similar to that of the wild-type strain. These data further confirm that complementation of the *rsbA*-defective mutant with the *rsbA* gene restored its responsiveness to resveratrol, and that inhibition of haemolysin expression by resveratrol was mediated mainly through RsbA in *P. mirabilis*.

#### Table 1. Effect of resveratrol on the cell-invasion ability of *P. mirabilis* strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Cell invasion (%)*</th>
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<tbody>
<tr>
<td>Wild-type</td>
<td>0·09 ± 0·01</td>
</tr>
<tr>
<td>Mutant†</td>
<td>0·21 ± 0·013</td>
</tr>
<tr>
<td>Wild-type + RV‡</td>
<td>0·02 ± 0·01</td>
</tr>
<tr>
<td>Mutant + RV</td>
<td>0·21 ± 0·012</td>
</tr>
</tbody>
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*Values represent the mean ± SD of three experiments.
†Mutant, *rsbA*-defective mutant.
‡RV, 30 µg resveratrol ml⁻¹.
the expression of virulence factors in prevention and treatment. Here we report that resveratrol, for their clinical potential, both in terms of disease
A wide variety of natural products has been under scrutiny
DISCUSSION
Fig. 4. Complementation of the rsbA-defective mutant of P. mirabilis by the wild-type rsbA gene restored wild-type responsiveness to resveratrol. The swarming migration distance (a) and the haemolysin activity (b) of different strains of P. mirabilis in the presence or absence of 60 μg resveratrol ml⁻¹ at various times after inoculation were determined. For haemolysin activity measurements, the value obtained with the wild-type cells in the absence of resveratrol at 4 h post-seeding was set at 100%. The data represent the means of three independent experiments. w, wild-type; m, rsbA-defective mutant; c, RsbA-complemented strain.

A wide variety of natural products has been under scrutiny for their clinical potential, both in terms of disease prevention and treatment. Here we report that resveratrol, a naturally occurring phytoalexin, can inhibit swarming and the expression of virulence factors in P. mirabilis. To our knowledge, this is the first report describing the inhibitory effect of resveratrol on bacterial swarming and virulence factor expression.

Resveratrol inhibits the growth of some bacterial species, but has little effect on others (Docherty et al., 2001; Mahady & Pendland, 2000; Tegos et al., 2002). Several lines of evidence indicate that the inhibitory effect of resveratrol on P. mirabilis swarming and virulence factor expression is unlikely to be due to its inhibitory effect on bacterial growth. Firstly, resveratrol inhibited P. mirabilis swarming and virulence factor expression at a concentration as low as 30 μg ml⁻¹ (Figs 1 and 3), but did not significantly affect the growth rate of the bacteria at concentrations up to 60 μg ml⁻¹ (Fig. 1c). Secondly, resveratrol did not affect the viability of P. mirabilis at a concentration of 256 μg ml⁻¹ (data not shown). Thirdly, while resveratrol could slightly inhibit the growth rate of both wild-type and rsbA-defective P. mirabilis, it could only inhibit swarming and virulence factor expression in the wild-type strain, and not in the mutant strain of P. mirabilis (Figs 1 and 3).

If resveratrol is to be used to treat or prevent P. mirabilis infection, its effect on human cells needs to be addressed. Our study showed that resveratrol inhibited P. mirabilis swarming and virulence factor expression at 30–60 μg ml⁻¹. At this concentration, resveratrol is expected to have little effect on normal human cells. For instance, the midpoint cytotoxicity values for a 24 h exposure to resveratrol are 98.6–105.4 μg ml⁻¹ for normal fibroblasts isolated from the oral cavity (Babich et al., 2000). Our study also indicated that human embryonal rhabdomyosarcoma cells treated with 30 μg resveratrol ml⁻¹ for 3 days did not show signs of cell death (data not shown).

Quorum sensing (QS) is a cell to cell communication mechanism that enables bacteria to monitor their own population density and synchronize the expression of virulence factors, and thus to mount effective attacks to overwhelm the host’s defence responses (Camara et al., 2002; de Kievit & Iglewski, 2000; Zhang, 2003). Although the regulation of virulence factor expression by QS is generally believed to be dependent on cell density, there is increasing evidence to suggest that the cell-to-cell signalling mediated by the QS system can also be strongly affected by environmental factors other than cell density. For example, bioluminescence, which is regulated by QS in Vibrio harveyi, has been found to be sensitive to the availability of iron, oxygen and carbohydrate (Nealson & Hastings, 1991). P. mirabilis swarming and virulence factor expression are generally believed to be regulated through a QS system (Holden et al., 1999; Schneider et al., 2002), and require the sensing and integration of a variety of environmental, cell-to-cell and intracellular signals. These signals may include those transmitted by high population density, autoinducer-2 (AI-2), cyclic dipeptides, putrescine, glutamine, intracellular cations and fatty acids (Allison et al., 1993; Holden et al., 1999; Lai et al., 1998; Liaw et al., 2004; Rather, 2005; Schneider et al., 2002; Sturgill & Rather, 2004). Recently, oxidative stress has been found to upregulate the expression of QS-controlled genes in Pseudomonas aeruginosa (Juhás et al., 2004; Kim et al., 2005). It is possible that oxidative stress also serves as an environmental signal to regulate QS in P. mirabilis. We propose that oxidative stress, as in Ps. aeruginosa, may also upregulate QS-regulated genes in P.
two-component signalling system (Belas RsbA, a His-containing phosphotransmitter of the bacterial (Ochsendorf, 1999).

The hypothesis that oxidative stress regulates P. mirabilis swarming is attractive, because it can explain the phenomenon by which swarming differentiation and virulence factor expression are dependent on cell density. Reactive oxygen species (ROS) are known to be generated physiologically during normal cell metabolism, and high cell density may lead to the accumulation of excessive ROS. At this stage, P. mirabilis starts to differentiate and migrate. As cells move out in swarming rafts, the concentration of ROS decreases, and eventually cells are unable to maintain the differentiated state and de-differentiate back to non-motile vegetative cells. During growth, ROS build up again, and differentiation/swarming proceeds for a second cycle. Regular cycles of differentiation/migration and de-differentiation/non-migration generate a colony on the agar surface with a characteristic pattern of concentric rings. That oxidative stress may cause bacteria to migrate has been reported previously (Nachin et al., 2005; Tang et al., 2004). It is also of interest to note that bacterial infections of the genital tract can lead to the production of excess ROS from neutrophils/macrophages as a first-line defence mechanism (Ochsendorf, 1999). P. mirabilis may sense this increase in ROS and migrate away from the attack by immune cells.

RsbA, a His-containing phosphotransmitter of the bacterial two-component signalling system (Belas et al., 1998; Liaw et al., 2001; Takeda et al., 2001), has previously been shown by us to act as a negative regulator of swarming differentiation and virulence factor expression in P. mirabilis (Liaw et al., 2001, 2004). Here, we have shown that resveratrol inhibits P. mirabilis swarming and virulence factor expression through an RsbA-dependent pathway. At least two models can explain the role of RsbA in resveratrol-mediated suppression of swarming and virulence factor expression. First, P. mirabilis may contain a two-component membrane sensor kinase which can sense the low-ROS condition that results from resveratrol treatment. Upon sensing low ROS, this kinase may transduce negative signals via the phosphotransmitter RsbA to inhibit swarming and virulence factor expression. Alternatively, P. mirabilis may contain a two-component sensor kinase which can sense the high-ROS condition and transduce signals to down-regulate the expression of RsbA, a negative regulator of swarming and virulence factor expression in P. mirabilis. Upon resveratrol treatment (which results in low ROS), the presumed sensor kinase can no longer transduce signals to inhibit RsbA expression, leading to accumulation of RsbA, and inhibition of swarming and virulence factor expression. To distinguish these two possibilities, we measured the expression of RsbA upon resveratrol treatment. Our preliminary data indicated that the production of RsBa mRNA in P. mirabilis was increased six- to sevenfold after resveratrol treatment. This result is more consistent with the second model.

Another possible mechanism by which resveratrol could inhibit P. mirabilis swarming and virulence factor expression is by acting as a QS signal mimic that disrupts the bacterial QS system. There is increasing evidence to suggest that plants can produce a variety of signal mimics that inhibit QS-regulated behaviours (Bauer & Robinson, 2002; Teplitski et al., 2000). For example, the halogenated furanones produced by the seaweed Delisea pulchra can specifically inhibit QS-regulated biological activities in various Gram-negative bacteria (Givskov et al., 1996; Hentzer et al., 2002; Teplitski et al., 2000; Zhang, 2003). The crude exudates from pea (Pisum sativum) and crown vetch (Coronilla varia) strongly inhibit the QS-regulated synthesis of violacein in Chromobacterium violaceum (Teplitski et al., 2000). That some plants can produce QS-inhibitory molecules after their long association with bacterial pathogens is not surprising, because in this way, hosts can evade the pathogenic effects of the bacteria. It is possible that resveratrol, a natural product of many plants (about 31 genera), also acts as a QS-inhibitory molecule to protect plants from bacterial infection. Since different bacterial species may use similar QS signals to communicate with each other, it is possible that resveratrol, although produced by plants, also acts on human pathogens to disrupt their QS systems.

The emergence of bacterial strains that exhibit resistance to various antibiotics poses a major threat to medicine and public health. As a consequence, there is renewed interest in antibacterial targets which, by attenuating virulence, disrupt the capacity of pathogenic bacteria to cause infection. In this context, the QS system is considered to be a potential therapeutic target, and the inhibition of QS may lead to control of infection.

In this paper, we demonstrate that resveratrol, an antioxidant found in some foods and drinks, has an inhibitory effect on P. mirabilis swarming and virulence factor expression. Although the molecular mechanisms underlying resveratrol inhibition of swarming and virulence factor expression are not clear, this finding opens up the opportunity to develop drugs that slow down P. mirabilis infection, allowing the host to gain valuable time to activate defence mechanisms, and to stop and eliminate pathogenic invaders.

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Resveratrol inhibits swarming and virulence