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New signaling molecules in some gram-positive and gram-negative bacteria

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Abstract

A new family of putative signaling molecules having a 2(5*H*)-furanone configuration has been described in this work. They were released during late exponential or stationary phase in different growth media by some gram-positive bacteria, such as *Lactobacillus helveticus*, *Lactobacillus plantarum*, *Lactobacillus paraplantarum*, *Lactobacillus sanfranciscensis*, *Enterococcus faecalis*, and a gram-negative species, i.e. *Salmonella enterica*. A pair of 2(5*H*)-furanones called furanones A and B occurred in all the conditioned media (CMs) of the species considered. These two molecules showed similar retention times and their spectral data shared the key fragments to include them in the 2(5*H*)-furanones family. However, some differences were observed in the mass fragmentation profiles. In particular the use of PCA analysis of all the mass fragments enabled the grouping of furanone A profiles of *S. enterica*, *L. helveticus*, *L. plantarum*, *L. paraplantarum*, *L. sanfranciscensis* and *E. faecalis* in one unique cluster with only few exceptions. On the other hand, the mass fragmentation profiles of furanone B of the major part of the species and strains could be grouped together and were differentiated from those of *L. helveticus*.

The specific activity of cell-free supernatants of high density cultures of *S. enterica* confirmed that the release of active molecules, and specifically of furanones A and B, was cell density dependent. Moreover, a preliminary experiment suspending *S. enterica* cells into cell-free supernatants of *L. helveticus* previously exposed to an oxidative stress demonstrated that furanones A and B have a strong interspecific activity. In fact cell autolysis and cell envelope damages were observed with Scanning Electron Microscopy (SEM) in *S. enterica*.

Keywords: 2(5*H*)-furanones; Lactobacilli; *Enterococcus faecalis*; *Salmonella enterica*; Conditioned media; Stress exposure

1. Introduction

Bacteria can produce an extensive array of secondary metabolites and can respond to a wide variety of chemicals in their environment. Particular groups of secondary metabolites have been characterised for their role in the regulation of gene expression in a cell density dependent manner according to the phenomenon that is nowadays referred to as quorum-sensing or cell-to-cell communication (Keller and Surette, 2006). In gram-negative bacteria, quorum-sensing typically involves an acylated homoserine lactone (AHL) autoinducer whose synthesis is dependent on a "LuxI" autoinducer synthase and a cognate "LuxR" autoinducer binding/transcriptional activator protein (Schauder et al., 2001). On the other hand, besides autoinducers of the ATP-binding cassette (ABC) transporter for secretion, the most common mechanism of quorum-sensing in gram-positive bacteria consists of a peptide and a two-component system for sensing the autoinducer concentration (Dirix et al., 2004). However, LuxS homologues associated with AI-2 synthase of gram-negative bacteria have also been reported for the genoma sequences of *Lactobacillus acidophilus* (Altermann et al., 2005), *Lactobacillus plantarum* (Kleerebezem et al., 2003), *Lactobacillus johnsonii* (Pridmore et al., 2004) and *Bifidobacterium longum* (Schell et al., 2005). In a recent work Ndagijimana et al. (2006) reported that two 2(5*H*)-furanones, in association with medium-chain fatty acids, were released by *Lactobacillus helveticus* exposed to oxidative and osmotic stresses. Experimental evidence of the involvement in the autolysis phenomenon of the two 2(5*H*)-furanones, detected by a gas chromatographic–mass spectrometry/solid phase microextraction technique, have also

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been obtained. New autolysins were detected concomitant with the exposure of *L. helveticus* to the cell-free conditioned media (CMs) containing the microbial furanones and to two commercial furanones having spectral data similar to those of the newly described 2(5*H*)-furanones (Ndagijimana et al., 2006). Moreover, cell morphological changes associated with exposure of *L. helveticus* to CMs containing the two furanones were observed. Therefore these molecules, which are volatile and have a presumptive molecular mass ranging between 143 and 180, meet a number of criteria proposed for the inclusion of a metabolite in the cell-to-cell signal molecules (Winzer et al., 2002).

In order to ascertain whether this new family of molecules is specifically associated to *L. helveticus* or it is more widespread in the bacterial world, various strains belonging to both gram-positive and gram-negative bacteria have been considered in order to evaluate: i) the volatile molecules released during the late exponential/stationary phase of their growth or in response to chemico-physical stresses; ii) the possible differences between the chemical configuration of the molecules having a potential signaling role. Moreover, a preliminary assessment of the concerted response generated in cells exposed to conditioned media containing the 2(5*H*)-furanones has been performed.

2. Materials and methods

2.1. Bacterial strains and culture conditions

L. helveticus CNBL 1156, obtained from the collection of the Istituto di Microbiologia, Universita` Cattolica del Sacro Cuore, Piacenza (Italy), *Lactobacillus sanfranciscensis* CB1, from the collection of the Istituto di Microbiologia Lattiero-Casearia, Università degli Studi di Perugia, Perugia (Italy), *L. sanfranciscensis* 77St, 201, 274, BB12, *L. plantarum* ATCC14917^T and *Lactobacillus paraplantarum* 4DE, from the collection of the Dipartimento di Scienze degli Alimenti, Università degli Studi di Teramo (Italy), were grown in MRS broth under anaerobic conditions (Anaerocult A; Merck, Darmstadt, Germany). Incubation temperatures were 44 °C for *L. helveticus*, 30 °C for *L. plantarum*, *L. paraplantarum* and *L. sanfranciscensis*. *Salmonella enterica* strain 155, from the collection of the Dipartimento di Scienze degli Alimenti, Università degli Studi di Bologna (Italy), and *Enterococcus faecalis* strain ORG1F from the collection of the Istituto di Microbiologia, Universita` Cattolica del Sacro Cuore, Piacenza (Italy), were routinely grown in Brain Heart Infusion (BHI, Oxoid) at 37 °C under aerobic conditions.

2.2. Preparation of conditioned media and exposure to stress condition

The media used for the preparation of the CMs and/or for stress exposure were: 1) whey obtained from a preparation of Parmigiano Reggiano cheese and sterilized by filtration as previously reported (Guerzoni et al., 2001) for *L. helveticus*; 2) wheat flour hydrolysed (WFH) broth (Gobbetti et al., 1994) for *L. sanfranciscensis*, *L. plantarum* and *L. paraplantarum*; 3) BHI for *S. enterica* and *E. faecalis*. In Table 1 the composition of the media used is reported.

Table 1
Composition (expressed as g/l) of media (BHI, whey and wheat flour hydrolysed — WFH) used for the preparation of the CMs and/or for stress exposure

BHI		Whey		WHF	
Calf brain	12.5	Lactose	54.0	Flour	100
Beef heart	5.0	Glucose	0.5	Yeast extract	3.5
Proteose peptone	10.0	Galactose	0.6	Maltose	7.5
Sodium chloride	5.0	Lactic acid	0.5	Glucose	7.5
Glucose	2.0			Fructose	2.5
Disodium hydrogen phosphate	2.5			Tween 80	0.3
pH	7.04	pH	6.5	pH	5.6–5.8

Each microbial species, grown overnight as preculture in the above reported media, was centrifuged and resuspended in fresh media (i.e., whey for *L. helveticus*, WFH for *L. sanfranciscensis*, *L. plantarum* and *L. paraplantarum*, and BHI for *S. enterica* and *E. faecalis*) and grown overnight at their optimal temperatures. The cells were then centrifuged for 10 min (10,000 × *g* at 4 °C), filtered (0.22 μm) and supernatants were used as conditioned media and for stress exposure.

Overnight cells of each microbial species were resuspended at concentrations $\geq 8.0 \pm 0.3$ log CFU/ml in the various CMs, obtained as above described, modified or not with the addition of H_2O_2 (0.017% v/v), sucrose (40% w/v) for *L. sanfranciscensis*, *L. plantaruman* and *L. paraplantarum*, NaCl (0.5 M) for *L. helveticus* and *E. faecalis* or chemical compounds, i.e. hexanal, alpha-angelica lactone, isovaleric acid (300 ppm). Hexanal and alpha-angelica lactone have been chosen on the basis of previous researches evidencing their antimicrobial activity, while isovaleric acid is a metabolite frequently released under stress conditions by lactobacilli (Guerzoni et al., 2007). After 2 h of exposure at the optimal temperature suspensions were centrifuged, filtered and analysed with gas chromatography–mass spectrometry/solid phase microextraction (GC–MS/SPME) in comparison to the original CMs. The data reported are the mean of four replicates.

2.3. Effect of the CMs from low density and high density cultures of S. enterica on the growth dynamics of active cells of the same species

S. enterica was cultured in BHI at 37 °C overnight. Cells were collected by centrifugation, washed twice in sterile saline solution and inoculated (3–4 log CFU/ml) in CMs of *S. enterica* whose growth had been interrupted when cells attained a level of about 6 log CFU/ml (low density) or 8 log CFU/ml (high density). The growth dynamics were analysed on the basis of plate counts onto agarized BHI incubated at 37 °C.

2.4. GC–MS/SPME analysis of volatile compounds

A divinylbenzene–carboxen–polydimethylsiloxane-coated fiber (65 μm) and a manual SPME holder (Supelco Inc.,