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Quinate metabolism and utilization as phenotypic properties to identify *Erwinia cypripedii* and *E. rhapontici*

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ABSTRACT

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Forty seven strains representing 8 species of plant pathogenic *Erwinia* were tested for quinate metabolism and utilization as a carbon source for growth. Both *E. cypripedii* and *E. rhapontici* had a capacity to metabolize quinate, tested on SQY (succinate-quinate-yeast extract) medium, but only *E. cypripedii* acquired a capacity to use quinate as a sole carbon source for growth. Strains of other *Erwinia* spp. tested neither metabolized quinate nor used it for growth. Thus, these two capacities can be used to differentiate *E. cypripedii* and *E. rhapontici* from other *Erwinia* spp. Southern hybridization analysis indicated that *E. cypripedii* and *E. rhapontici* harbored a homologue of *qum*A, encoding pyrrolo-quinoline quinone-dependent quinate dehydrogenase. Quinate metabolism and *qum*A gene have previously been reported to be specific to pathovars in DNA homology group 6 of *Xanthomonas campestris*. Therefore, the limited distribution of *qum*A among xanthomonads and erwiniae indicated that DNA group 6 xanthomonads, *E. cypripedii*, and *E. rhapontici* might acquire *qum*A through a horizontal gene transfer.

Keywords: quinate; *qum*A; *Erwinia*

INTRODUCTION

Quinate metabolism is a phenotypic property specific to DNA homology group 6 of *Xanthomonas campestris*, comprising at least 140 pathovars that are differentiated by their ability to cause disease on a particular plant or plants [1,6]. The group 6 includes *X. campestris* pv. *celebensis*, *X. campestris* pv. *corylina*, *X. campestris* pv. *juglandis*, and *X. campestris* pv. *pruni*, which has been reclassified into a new species *X. arboricola* by Vauterin *et al.* [11]. Although xanthomonads in group 6 can metabolize quinate, they cannot utilize it as a sole carbon source for growth. A *qum*A gene involved in quinate metabolism has been cloned from *X. campestris* pv. *juglandis* and the gene also shows genomic specificity to xanthomonads in group 6 [7]. The DNA sequence of *qum*A indicates that the gene encodes a quinate dehydrogenase, a member of a family of pyrrolo-quinoline quinone (PQQ)-dependent quinate dehydrogenase [7]. Because of the limited distribution pattern of quinate metabolism and *qum*A among xanthomonads, xanthomonads in group 6 may acquire *qum*A from other plant pathogenic or non-pathogenic bacteria through a horizontal gene transfer, and may not inherit the gene from a common ancestor of xanthomonads. To gain further information on the distribution of quinate metabolism and utilization capacities in other bacteria, we first tested the capacities among plant pathogenic erwiniae. The results showed that the capacities were limited to two species of Erwinia, *E. cypripedii* and *E. rhapontici*, and made the quinate metabolism and utilization tests very useful for the identification and delineation of these two *Erwinia* species.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and DNA manipulations

The bacterial strains used in this study are listed in Table 1. *Erwinia* strains and *Escherichia coli* were routinely cultured on Luria-Bertani agar (LA) or broth medium [10]. Mini-scale preparations of *E. coli* plasmid DNA, total genomic DNA isolation of plant pathogenic bacteria, restriction endonuclease treatments, and agarose gel electrophoresis were done as described by Sambrook and Russell [10]. A number of named erwiniae were obtained from

Table 1. Quinate metabolism and utilization tests of *Erwinia* spp.

Species or subspecies	Strains tested	Alternative genus name [4]	Cluster [5]	SQY [6]	QY [7]
E. amylovora [1]	EA137, EA169	*Erwinia*	II	-	-
E. carotovora subsp. *atroseptica* [1]	UCBPP148,UCBPP149	*Pectobacterium*	III	-	-
E. carotovora subsp. *betavasculorum* [1]	UCBPP162, UCBPP163	*Pectobacterium*	III	-	-
E. carotovora subsp. *carotovora*		*Pectobacterium*	III	-	-
from chinese cabbage [2]	Erc1, Erc3				
from potato [3]	CCRC 13159				
from calla lily [2]	ErcS3-1, ErcS3-2				
E. chrysanthemi		*Pectobacterium*	III	-	-
from green onion [3]	CCRC 12616				
from celery [3]	CCRC 13147				
from *Phalaenopsis* [2]	EchP3, EchL8				
from white-flowered calla lily [2]	EchS3-1, EchH1, EchH3				
E. chrysanthemi pv. *dieffenbachiae* [1]	ED102B, ED104	*Pectobacterium*	III	-	-
E. chrysanthemi pv. *paradisiaca* [1]	EC226, EC227	*Pectobacterium*	III	-	-
E. chrysanthemi pv. *parthenii* [1]	EC110, EC196	*Pectobacterium*	III	-	-
E. chrysanthemi pv. *zeae* [1]	EC209, EC210	*Pectobacterium*	III	-	-
E. nigrifluens [1]	EN104, EN105	*Brenneria*		-	-
E. quercina [1]	EQ101, EQ102	*Brenneria*		-	-
E. rubrifaciens [1]	ER103, ER104	*Brenneria*		-	-
E. rhapontici [1]	ER1, ER102, ER106, ER107, ER108, ER110, UCBPP1051, UCBPP1052, UCBPP1053	*Erwinia*	II	+	-
E. cypripedii [1]	EC115, EC160, EC162, EC163, EC164, EC168	*Pectobacterium*	II	+	+

1. The strains were obtained from the International Collection of Phytopathogenic Bacteria, University of California, Berkeley, U.S.A.
2. The strains were isolated from plant hosts in Taiwan, and collected in Department of Life Science, Fu Jen Catholic University, Taiwan.
3. The strains were purchased from Bioresources Collection and Research Center, Hsinchu, Taiwan.
4. The alternative genus names of *Erwinia* spp. were based on Hauben *et al*. (1998).
5. The clusters were defined by Kwon *et al*. (1997).
6. The positive strains in SQY (succinate/quinate/yeast extract medium) test produce a deep-green color around the bacterial streak, and negative strains have no color developed.
7. The positive strains in QY (quinate/yeast extract medium) test can grow on the medium, and negative strains cannot.

the International Collection of Phytopathogenic Bacteria (ICPB), University of California, Berkeley.

Quinate Metabolism and Utilization Tests

For testing quinate metabolism, the succinate/quinate/yeast extract (SQY) was used. The SQY medium per liter contained 10 g succinic acid (disodium salt, hexahydrate), 5 g quinic acid, 1.5 g K_2HPO_4, 1.0 g $(NH_4)_2SO_4$, 0.5 g yeast extract, 15 g agar. The pH was adjusted to 7.2-7.5 with 10 N NaOH and autoclaved for 15 minutes at 121℃. After autoclaving, 7.5 ml of autoclaved 20% $MgSO_4 \cdot 7H_2O$ solution was added to the medium to give a final concentration of 0.15%. Four to six bacterial cultures were streaked per plate and incubated for 4 to 6 days at 28℃. The diffusion of a deep- green color around a bacterial streak, indication of the breakdown of quinate, was considered as a positive reaction [(6)]. For testing quinate utilization capacity, quinate/yeast extract (QY medium) was used instead of SQY medium. The QY medium had the same composition as SQY medium except that succinic acid was omitted. The growth of bacterial cultures on the medium was considered as a positive reaction. Plates of SQY and QY media could be stored at 4℃ in plastic bags for at least one month without losing their effectiveness.

Southern blot analysis

Southern blots were performed as described by Sambrook and Russell [(10)] using a digoxigenie (DIG) DNA labeling and detection kit (Boehringer-Mannheim, Mannheim, Germany). A DIG-labeled DNA probe was prepared from pQM38 carrying the *qum*A gene of *X. campestris* pv. *juglandis* XJC5 [(7)] using PCR DIG probe synthesis kit (Boehringer Mannheim, Mannheim, Germany). The PCR for preparation of DIG-labeled DNA probes was performed in an air thermal cycler (Rapidcycler, Idaho Technology, ID) programmed for denaturation at 94℃ for 2 min and then for 35 cycles of 2 sec at 94℃, 2 sec at 50℃, and 35 sec at 72℃. Hybridizations were carried out with a hybridization temperature of 68℃ and two 10 min washes with 0.1x SSC and 0.1% sodium dodecyl sulfate at 68℃ according to the manufacturer's protocol.

RESULTS AND DISCUSSION

Quinate metabolism and utilization tests

Erwinia species positive for the SQY test were detected only in *E. cypripedii* and *E. rhapontici*. All strains of *E. cypripedii* and *E. rhapontici* were positive and produced a deep-green color around the bacterial streak, indicative of the breakdown of quinate. Strains of other *Erwinia* species could grow on SQY medium but no colored developed. In succinate-free QY medium, *Erwinia* species tested could not utilize quinate as a sole carbon source for growth with the exception of strains of *E. cypripedii*. All strains of *E. cypripedii* tested could grow on QY medium (Table 1). The results indicated that among *Erwinia* spp. tested, both *E. cypripedii* and *E. rhapontici* can metabolize quinate, and only *E. cypripedii* can utilize quinate as a carbon source for growth. Thus, these two capacities can be used to differentiate *E. cypripedii* and *E. rhapontici* from other *Erwinia* species.

E. cypripedii and *E. rhapontici* can grow and produce a deep-green color on a SQY medium. However, although *E. cypripedii* can grow on a QY medium, it did not produce a deep-green color. The reason for the failure of color development may be the pH value. Since the color development in the SQY medium is the result of the accumulation of gallic acid which is a deep-green color above pH 7, pH below 7.0 will not support color development [6]. The pH in SQY media increased to about 9.5 after 2-3 day inoculation. However, the pH in QY decreased to about 4.5 after growth of bacterial strains.

The universal expression of quinate metabolism among all tested strains of *E. cypripedii* and *E. rhapontici* and its general absence among other *Erwinia* spp. tested makes the quinate metabolism by a SQY test very useful for the identification of *E. cypripedii* and *E. rhapontici*. In addition, utilization of quinate as sole carbon source by a QY test can further be used to delineate between *E. cypripedii* and *E. rhapontici*.

E. cypripedii can cause rotting symptoms on orchid (*Cypripedium* spp.) and papaya [8,9], and *E. rhapontici* is a causal agent of pink seed and crown rot or soft rot of rhubarb (*Rheum rhaponticum* L.) and other plant species [4]. *E. cypripedii* and *E. rhapontici* belong to the 'carotovora group', which includes bacteria that produce pectolytic enzymes and cause soft rot in plants, but they are unable to degrade pectate [2]. In this study, two more abilities, quinate metabolism and utilization, were demonstrated to be very useful to distinguish *E. cypripedii* and *E. rhapontici* from other *Erwinia* species.

In addition to identification, quinate metabolism may have taxonomic significance in differentiating *Erwinia* spp. Phylogenetic studies using 16S rRNA gene sequences have shown that both *E. cypripedii* and *E. rhapontici* belong to the same cluster [5]. This close relationship may imply that *E. cypripedii* and *E. rhapontici* originate from a common ancestor. The capacity of quinate metabolism of *E. cypripedii* and *E. rhapontici* was inherited from a common ancestor and have been resident in these bacteria before their separation. Alternatively, the occurrence of horizontal transfer between these two bacteria in the past could be another explanation.

Southern hybridization

An internal fragment of *qum*A, encoding PQQ-dependent quinate dehydrogenase [7], was used as a probe to hybridize *Hin*dIII-digested total DNA from strains of *Erwinia* spp. tested. The *qum*A probe hybridized to the DNA from all strains of *E. cypripedii* and *E. rhapontici*, but not to the DNA of strains from other *Erwinia* spp. tested. This result suggested that *E. cypripedii* and *E. rhapontici* also harbored a homolog of *qum*A, and the homolog is limited to *E. cypripedii* and *E. rhapontici* among *Erwinia* spp. tested.

Although the *qum*A probe hybridized with genomic DNAs of all strains of *E. cypripedii* and *E. rhapontici* tested, the size of the hybridized fragment is different between *E. cypripedii* and *E. rhapontici*. The probe hybridized to a *Hin*dIII fragment larger than 12.0 kb in strains of *E. cypripedii*, but to a 4.0-kbp *Hin*dIII fragment in strains of *E. rhapontici*. Representative results are shown in Fig. 1.

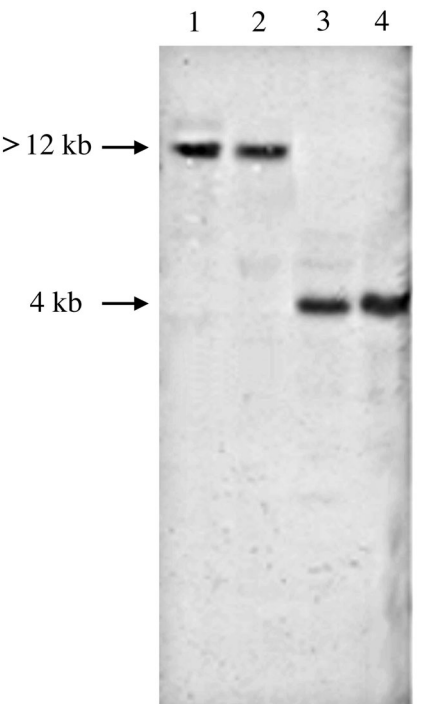

Fig. 1. Southern hybridization of *Hin*dIII-digested total DNA from *Erwinia cypripedii* and *E. rhapontici* using an internal fragment of qumA as a probe. Lanes: 1, *E. cypripedii* EC155; 2, *E. cypripedii* EC168; 3, *E. rhapontici* ER102; 4, *E. rhapontici* ER106. DNA (0.5 μg) was loaded into each lane, electrophoresed in 1% agarose, transferred to a Nytran membrane (0.45- μm pore size; Schleicher & Schull, Keene, NH), and probed with the DIG-11-dUTP-labeled probe. The sizes of the bands are indicated on the left.

The limited distribution pattern of quinate metabolism and *qum*A homologs among xanthomonads and erwiniae may lead to the proposal that *qum*A can be horizontally transferred among *X. arboricola*, *E. cypripedii*, and *E. rhapontici*. Although no solid evidence to substantiate it so far, it will be interesting to further determine how horizontal gene transfer occurs in plant-associated bacteria and how it contributes to the evolution of bacterial pathogenic interactions with plants.

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摘 要

李永安[1,2]、周淑玲[1]. 2003. 奎寧酸代謝及利用的表現性狀可用以鑑定*Erwinia cypripedii* 及*E. rhapontici*. 植病會刊 12:242-246. ([1] 輔仁大學生命科學系; [2] 聯絡作者，電子郵件：bio1007@mails.fju.edu.tw；傳眞：+886-2-2902-1124)

測試 *Erwinia* 屬內的八個種共 47 株菌株的代謝奎寧酸以及利用奎寧酸爲生長唯一碳素源的能力，結果發現 *E. cypripedii* and *E. rhapontici* 具有代謝奎寧酸的能力，而只有 *E. cypripedii* 能利用奎寧酸爲生長的唯一碳素源，而其餘 *Erwinia* 屬的六個種則不具代謝及利用奎寧酸的能力。因此，代謝及利用奎寧酸的兩個能力可用以鑑定及區分 *E. cypripedii* 及 *E. rhapontici*。南方氏雜合反應結果顯示 *E. cypripedii* 及 *E. rhapontici* 含有一個 *qum*A (奎寧酸去氫酶基因) 的同源基因，先前研究結果發現在 *Xanthomonas* 屬內，奎寧酸代謝能力及 *qum*A 只存在於 *Xanthomonas* DNA homology group 6 的病原小種內，因此，*qum*A 的局限分布情形表示 *qum*A 可能經由水平轉移方式移入 *Xanthomonas* 及 *Erwinia* 屬內的少數幾個種內。

關鍵詞：奎寧酸、奎寧酸去氫酶基因、軟腐病菌