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OEM—A new medium for rapid isolation of onion-pathogenic and onion-associated bacteria

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ABSTRACT

Onions (*Allium cepa* L.) are plagued by a number of bacterial pathogens including *Pantoea ananatis, P. agglomerans, Burkholderia cepacia, Enterobacter cloacae, Pectobacterium carotovorum* subsp. *carotovorum, Xanthomonas axonopodis* pv. *axonopodis* and several *Pseudomonas spp.* We developed a semi-selective medium, termed onion extract medium (OEM), to selectively and rapidly isolate bacteria pathogenic to and associated with onions and onion-related samples including bulbs, seeds, sets, transplant seedlings, soil and water. Most strains of interest grow sufficiently on OEM in 24 h at 28 °C for tentative identification based on colony morphology, facilitating further characterization by microbiological and/or molecular means.

1. Introduction

Onion (*Allium cepa* L) is an important vegetable crop. According to the USDA Economic Research Service, more than 72 million metric tons of onions were produced in the United States in 2008; the United States crop alone was valued at more than 800 million US dollars. Bacterial diseases of onions are of major economic concern, with losses occurring in the field as well as during storage. In years with weather conditions favoring bacterial growth during the onion-growing season, growers estimate losses of up to 40% (Beer et al., 2012).

Several bacterial diseases of onions are problematic for onion growers around the world because they cause foliage problems and losses to bulbs at harvest, during storage and in the marketing chain (Schwartz and Mohan, 2008). Bulb pathogens often cause indistinct symptoms, yet they are responsible for substantial losses. Often the problems are termed “bacterial decay” or just “rot.” Because the symptoms caused by the several different pathogens are not distinctive, identification of the pathogen is necessary. The first step towards identification generally involves isolating the bacterium in pure culture, which instigated these studies. Identification is important because different pathogens elicit different symptoms and the epidemiology of each likely differs from that of others and therefore strategies for their management differ.

2. Bacterial pathogens of onion and media for isolation

2.1. Bacterial pathogens

Among the more important bacterial pathogens affecting onion bulbs are *Burkholderia cepacia, Pantoea ananatis* and *Enterobacter cloacae*. Sour skin, described initially as caused by *Pseudomonas cepacia* (Burkholder, 1950), and lately renamed *B. cepacia*, is perhaps the most common and important bulb disease. Often it affects onions near or at harvest, and the pathogen generally enters the plant through wounds resulting from wind-driven hail or tillage and harvesting equipment or excessive and prolonged excessive moisture conditions (Schwartz and Mohan, 2008). Center rot caused by *Pantoea ananatis*, was observed first by Gitaitis and Gay (1997) in Georgia, USA. Shortly thereafter, it was reported in onion-growing areas of Colorado, Michigan and New York (Schwartz and Otto, 2000; Gitaitis et al., 2002; Carr et al., 2010). The pathogen is postulated to have traveled from South Africa to the US on onion seed, and natural infestation of onion seed by *P. ananatis* has been reported (Walcott et al., 2002 and Goszczynska et al., 2006a). Recently, the disease was reported in Korea (Kim and Choi, 2012). The disease begins in the field where central leaves are affected by blight; leaf lesions progress to neck and then to the connected scales within the bulb (E.A. Carr, A.M. Zaid, J.M. Bonasera and S.V. Beer, unpublished data). Enterobacter bulb decay caused by *Enterobacter cloacae* (Jordan) has been reported in Australia (Cother and Dowling, 1986), California (Bishop and Davis, 1990), Colorado (Schwartz and Otto, 2000), Washington (Schroeder et al., 2009, 2010) and most recently in New York (Zaid et al., 2011). *B. cepacia* and *E. cloacae* rarely cause foliar symptoms, but they excel at rotting bulbs (Schwartz and Mohan, 2008).

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Xanthomonas axonopodis pv. *allii*, which causes a bacterial leaf blight of onions, was recorded in Hawaii (Alvarez et al., 1978), then Brazil (Neto et al., 1987), and later from several states of the continental United States (Isakeit et al., 2000; Schwartz and Otto, 2000; Nunez et al., 2002; and Sanders et al., 2003). The blighting reduces photosynthetic efficiency and results in plant stunting and reduced bulb size (Gant and Schwartz, 2008).

Numerous other species of pathogenic bacteria, for example, *Pectobacterium carotovorum, Dickeya chrysanthemi, Pseudomonas viridiflava* and some strains of *Pantoea agglomerans,* also can cause disease and substantial economic losses in onions in some areas on a sporadic basis (surveyed by Schwartz and Mohan, 2008). An improved understanding of the life styles of the various onion pathogens, which begins with their identification, will be helpful in developing effective management and control strategies. OEM was developed to that end.

2.2. Media for isolation

Many general media such as nutrient agar (NA), LB agar, tryptic soy agar, yeast extract peptone agar (YP), and yeast extract calcium carbonate agar (YDC) have been used to isolate onion-pathogenic bacteria from onion tissues (Azad et al., 2000; Coutinho et al., 2002; Coutinho and Venter, 2009; Walcott et al., 2002; Kido et al., 2008, 2010). However, these media are not selective, and organisms present as saprophytes in plant materials may interfere with growth of target bacterial pathogens of onions.

Several semi-selective media for individual onion pathogens have been reported. For example, PA-20 was developed for isolation and enumeration of *P. ananatis* from onion seeds (Goszczynska et al., 2006b). However, incubation at 25 °C for 6–7 days is required before *P. ananatis* develops sufficiently for colony characterization. Neomycin, Cephalexin, Trimethoprim, Pivmecillinam (NCTM 1) medium was developed as a semi-selective media for isolation of *Xanthomonas* spp. from contaminated onion seeds (Roumagnac et al., 2000). PCAT and TB-T media were developed by Lumsden and Sasser (1986) and Hagedorn et al. (1987), respectively, for isolation of *B. cepacia*. None of the mentioned semi-selective media are reported to support growth of most onion-pathogenic and onion-associated bacteria.

We aimed to develop a semi-selective medium to isolate, characterize and presumptively identify all mentioned onion-pathogenic bacteria from onions and onion-associated materials. Our goal was to develop a selective medium that would permit the rapid isolation of bacteria in preparation for rigorous identification and pathogenicity studies.

3. Materials and methods

3.1. Bacterial strains

Bacterial strains used in this study are listed in Table 1. Stock cultures of strains were maintained in 15% (v/v) glycerol in water at −80 °C. Stock strains were streaked onto LB agar plates at 28 °C to recover growing cultures. Cultures were routinely checked for purity and colony characteristics.

3.2. Development of onion extract medium (OEM)

Onion extract was the main source of nutrients for OEM. The pH of the medium was adjusted to 5.8 by addition of potassium phosphate salts, and sodium chloride was added to increase osmotic concentration. Crystal violet was added to suppress the growth of Gram-positive bacteria (Ishimaru and Klos, 1984), and the medium was supplemented with cycloheximide to retard or prevent the growth of mycelial fungi and yeasts (Cyzeska et al., 1981; Kawanishi et al., 2011).

The medium was prepared as follows: peeled yellow onions (333 g) were chopped, autoclaved in ca. 500 ml of high-purity (HP) water for 35 min at 121 °C, cooled to room temperature, then filtered through coarse filter paper (CAT # 28331–081 VWR Scientific Products, West Chester, PA 19380). The following were added to the filtrate: 5 g NaCl, 1 g of K_2HPO_4 (anhydrous), 3.8 g of KH_2PO_4 (anhydrous), 2.5 ml of crystal violet stock solution (75 mg/100 ml) and 250 mg of cycloheximide. The volume was made up to 1 L by addition of HP water. The pH was checked and adjusted to 5.8, if needed, by adding KH_2PO_4 or K_2HPO_4. Agar (18 g) was added before autoclaving at 121 °C for 35 min. The medium was cooled to 55 °C and poured into 15 mm-deep X 100 mm-diameter plastic Petri plates (25 ml/plate).

3.3. Colony morphology and plating efficiency of some onion-pathogenic bacterial strains on OEM

Strains were grown on LB agar plates for 24 h at 28 °C. Colonies were suspended in sterile HP water to $OD_{600nm} = 0.2$. Each bacterial strain was dilution-streaked on OEM plates to obtain single colonies. The morphological and growth characteristics of single colonies on OEM were described.

To determine plating efficiency of OEM, bacterial suspensions were prepared in sterile water to $OD_{600nm} = 0.2$. Ten-fold serial dilutions were prepared to 10^{-5}. Seven replicate 5 µl drops of the 10^{-4} and/or 10^{-5} dilutions were applied to the surface of OEM and LB agar plates for each bacterial strain. Plates were incubated at 28 °C for 24 h. Bacterial growth was calculated as cfu per 5 µl for each strain. Colony morphology was checked with the aid of a dissecting microscope to reduce the possibility of counting contaminants. Plating efficiency was calculated based on mean number of cfu on OEM divided by mean cfu on LB X 100. This experiment was repeated several times with similar results.

3.4. Assessment of selectivity of OEM

3.4.1. Growth characteristics of onion-pathogenic and onion-associated bacterial strains on OEM

Bacterial strains were dilution-streaked from −80 °C glycerol stocks onto LB plates and incubated at 28 °C for 24 h. Bacterial suspensions were prepared to achieve $OD_{600nm} = 0.2$, and one loopful from each bacterial suspension was streaked on OEM plates. Plates of OEM were incubated at 28 °C and characteristics of the resulting colonies were compared with those growing on LB agar plates.

3.4.2. Growth rate of onion-pathogenic and onion-associated microorganisms in OEM Broth

Seven microorganisms including three onion-pathogenic bacteria (*P. ananatis, B. cepacia* and *E. cloacae*), three onion-associated microbes isolated from New York onions and tentatively identified based on amplification of a fragment of *gyrB gene* (*Ewingella* sp. strain A14a, *Bacillus megaterium* strain M66 and *Pichia* sp. strain H8c), were tested for their growth rate in Onion Extract Broth containing all the components of OEM except agar.

Fresh cultures of all bacterial strains and *Pichia* sp. were streaked on LB agar plates from glycerol stock maintained at −80 °C. The microbes were incubated at 28 °C for 24 h, then a suspension from each culture was prepared in sterile HP water to achieve $OD_{600nm} = 0.2$. Fifty microliters from each suspension was inoculated into 5 ml of OEM broth, in triplicate, for each strain and incubated at 28 °C with shaking at 200 rpm. Growth rate was monitored for 12 h based on OD_{600nm} at 2-h intervals.

3.4.3. 3. Isolation of onion-pathogenic bacteria from artificially infected bulbs

Onion bulbs considered free of bacterial pathogens were artificially inoculated with *P. ananatis* CU6829, *B. cepacia* CU6878, *E. cloacae* CU6882, or *P. agglomerans* CU2019. Inoculum of each bacterium was prepared as previously described. Each bacterial suspension (0.3 ml)

Table 1
Bacterial strains: their sources, growth on OEM and LB and their pathogenicity to onion leaves and/or onion sets.

Bacterial strain	CU code*	Original code or isolator	Source	Growth on OEM	Growth on LB agar	Pathogenicity test in onion leaves and/or sets
Pantoea ananatis	CU6790	Pan 97-1	Onion-GA	+	+	+
	CU6829	OC5a	Onion-NY	+	+	+
	CU6811	BD 647	Maize-South Africa	+	+	+
	CU6758	Pan 99-9	Verbena-GA	+	+	−
	CU6818	LMG 2665	Pineapple-Brazil	+	+	−
	CU6835	OC42	Onion-NY	+	+	−
	CU6810	BD640	Maize-South Africa	+	+	−
	CU6822	LMG 20103	Eucalyptus-South Africa	+	+	+
	CU6366	Hort.Hill 31**	Onion Seed-GA	+	+	+
	CU6313	Hort Hill 24**	Onion Seed-GA	+	+	+
	CU6314	Black Shank 15**	Onion Seed-GA	+	+	+
Pantoea agglomerans	CU2019	SUH2	Onion-South Africa	+	+	+
	N/A	AZ-48	Onion-NY (this study)	+	+	+
	N/A	AZ-52	Onion-NY (this study)	+	+	+
P. stewartii	CU0176	Woods (isolator)	Sweet Corn	+	+	NT
Burkholderia cepacia	CU3372	ATCC 17759	Soil-Trinidad	+	+	+
	CU0318	64-22NS	Onion	+	+	+
	CU6876	This study	Soil of onion-NY	+	+	+
	CU6877	This study	Soil of onion-NY	+	+	+
	CU6878	Christy IV	Soil of onion-NY	+	+	+
	CU3368	ATCC25416	Onion-NY	+	+	+
	CU2975		Soil, Mexico	+	+	+
Pseudomonas gladiola pv. alliicola	CU3891	ATCC19302	Onion	+	+	+
Pseudomonas gladioli pv. gladioli	CU3890	ATCC10248	Gladiolus	+	+	NT
Enterobacter cloacae	CU6881	ATCC23355	Onion, Columbia	+	+	+
	CU6882	ECWSU1=310	Onion, Washington State	+	+	+
	CU6883	ATCC13047	Spinal fluid Type strain CDC	+	+	+
	CU0295	204/77	CDC	+	+	+
	CU0296	280/77	CDC	+	+	+
	CU0297	ATCC29941	CDC	+	+	+
	CU6930	ECL2	Onion-NY (this study)	+	+	+
	CU6931	ECL3=ATCC BAA-2271	Onion soil-NY (this study)	+	+	+
P. carotovorum sub sp. carotovorum	CU4148	ATCC15713	Potato-Denmark	+	+	−
Pseudomonas sp.	CU4236	–	Onion bulb-Ontario	+	+	+
P. glycinea	CU3395	race-4	–	+	+	NT
P. putida	N/A	S22	Onion soil	+	+	−
Pseudomonas fluorescens	N/A	AZ-55	Onion-NY	+	+	+
P. marginalis	N/A	P79-C	Cull onions	+	+	NT
P. viridiflava	CU6938	20345 Ps. 175	Onion CAL, US	+	+	NT
P. glycinea	N/A	J45a		+	+	NT
P. putida	N/A	S22.a	Onion soil	+	+	−
Serratia marcescens	CU 0155	Strain 101	Steve Beer	+	+	NT
E. coli	CU2475	DH5α™	Invitrogen Life Technologies Grand Island, NY 14072 USA	+/−	+	−
E. amylovora	CU0273	ATCC49946	Apple-NY	+/−	+	NT
B. subtilis	CU0187	ST168	Isolater A.K. Vidaver	−	+	NT
B megaterium	N/A	M 66	Cull onions	−	+	NT
C. michiganesis sub sp. michiganesis	CU5761	99-01	Q. Shi	−	+	NT
Pichia sp.	N/A	H8c	Cull onions	−	+	NT
S. scabies	N/A	90-035***		−	+	NT
Ewingella sp.	N/A	A14a	Cull onions	+	+	+

+, growth sufficient after 24 h, or positive for pathogenicity in onion sets and/or onion leaves.
−, did not grow after 24 h of incubation at 28 °C or negative for their pathogenicity in onion sets/and/or leaves.
+/−, rarely grew following 24 h of incubation.
NT, not tested for pathogenicity.
* CU code; Culture Collection of Department of Plant Pathology and Plant-Microbe Biology, Cornell University, Ithaca, New York.
** Walcott, R. R., Gitaitis, R. D., Castro, A. C., Sanders, F. H., Jr., and Diaz-Perez, J. C. 2002. Natural infestation of onion seed by Pantoea ananatis, causal agent of center rot. Plant Dis. 86:106–111.
*** Plant Pathology Teaching Culture Collection, Cornell University, Ithaca, New York.

was injected with a sterile 26-gauge needle and 1-ml syringe into the middle of surface-disinfested onion bulbs. The inoculated bulbs were incubated for 1 week at 28 °C (*P. ananatis*, *B. cepacia* and *P. agglomerans*) or 37 °C (*E. cloacae*). After 7 days, all bulbs were cut longitudinally to observe symptoms of decay, discoloration and/or maceration on the internal scales. Symptomatic tissue was mashed using sterile wooden applicators and streaked directly on OEM plates. The plates were incubated for 24 h at 28 °C, and the resulting colonies were investigated for their growth and morphology. The isolated bacterial strains were re-streaked on LB agar plates and identified using standard biochemical tests (oxidase, indole production, KOH, nitrate reductase and fluorescence on King's B plates) and sequencing of a fragment of the *gyrB* gene (Bonasera et al., unpublished data).

3.4.4. Isolation from symptomatic naturally infected onion bulbs, necks and leaves

Symptomatic onion bulbs, leaves and leaf stalks (necks), collected from onion fields and several farm storages in New York, were examined visually for symptoms of bacterial disease. Bulbs were cut longitudinally and examined for internal decay, discoloration and maceration. Onion leaves were examined for blighting and/or discoloration; onion leaf stalks were cut longitudinally and examined for

discoloration and maceration. Small portions of symptomatic samples were transferred to sterile micro-centrifuge tubes containing 1 ml of sterile HP water. Each specimen was mashed using a sterile wooden applicator; the resulting suspension was streaked directly on the surface of OEM plates. The plates were incubated for 24 h at 28 °C. Resulting single colonies were examined for morphological characteristics and re-streaked on LB agar plates for further purification and identification using biochemical and molecular tools.

3.4.5. Isolation from onion transplants

3.4.5.1. Determination of epiphytic bacteria. Ten transplants (approximately 6-weeks-old) were washed by shaking in a plastic bag with 100 ml of sterile water for 30 min at room temperature. Serial 10-fold dilutions of the wash water were prepared in sterile water and 100 µl from each dilution was spread separately on OEM plates. The plates were incubated at 28 °C for 24 h before colonies were examined and counted. Representative colonies were re-streaked on LB agar plates for further purification, identification and pathogenicity testing.

3.4.5.2. Determination of endophytic bacteria. The 10 previously washed transplants were surface-disinfested by replacing the wash water with 100 ml of 15% (v/v) household bleach (6.25% sodium hypochlorite) in sterile water. After shaking for 10 min, the bleach solution was discarded and plants were washed twice in 100 ml of sterile water and then homogenized in 200 ml of sterile water in an electric blender. The homogenate was filtered through sterile filter paper (CAT # 28331–081 VWR Scientific products, West Chester, PA 19380, which is described as, "ideal for gelatinous and coarse precipitates"). One hundred microliters of undiluted and 10-fold diluted filtrate were plated on OEM. Plates were incubated at 28 °C for 24 h prior to examining and counting bacterial colonies. Sample colonies were streaked on LB agar plates for purification, further identification and pathogenicity testing.

3.5. Isolation from onion seeds

3.5.1. Removal of onion seed pelleting material

Samples were de-pelletized mechanically by rolling the pelleted seeds between the surface-disinfested laboratory bench top and a surface-disinfested wooden board with hand-applied pressure sufficient to shatter the pellets. The pelleting material was separated from the seeds using a sieve; the pelleting material passed through the sieve, while the onion seeds were retained.

3.5.2. Determination of epiphytic bacteria from onion seeds

One gram of de-pelletized seeds was suspended in 10 ml of sterile water in a 15-ml sterile plastic centrifuge tube. Tubes were shaken for 30 min on an electric shaker at room temperature. Ten-fold serial dilutions were made to 10^{-3}. One hundred microliters of each dilution was spread on the surface of OEM, followed by incubation at 28 °C for 24 h. Total bacterial counts were calculated as cfu per gram of seed. Bacterial colonies obtained from OEM plates were streaked on LB agar plates for single colony production and further identification and pathogenicity tests.

3.5.3. Determination of endophytic bacteria from onion seeds

The washed seeds were then surface-disinfested using 10 ml of 15% (v/v) household bleach (6.25% sodium hypochlorite) in sterile water and shaking for 10 min. The bleach was discarded and seeds were washed twice in 10 ml of sterile water. The final wash water (100 µl) was spread on the surface of OEM plates to test the efficiency of the surface disinfestation method. Seeds were then ground in 10 ml of sterile water in a sterile mortar and pestle. Ten-fold dilutions were made to 10^{-3} and 100 µl from each dilution were spread on the surface of OEM plates. Plates were incubated at 28 °C for 24 h. Total bacterial counts were calculated as cfu/g of seed, as prepared. Distinct single colonies were streaked on LB agar plates for purification, further identification and pathogenicity tests.

3.6. Isolation from soil

One gram of each soil sample was suspended in 10 ml of sterile water, shaken at room temperature for 1 h, and then filtered through sterile Whatman No. 1 filter paper to remove large particulates. Next, the aqueous portion was passed through 3-µm pore-size ultrafiltration membrane (Millipore) to remove smaller particles, fungal spores and hyphae. Each sample was diluted 1:1,000 with sterile water and plated on the surface of OEM. Following incubation at 28 °C for 24 h, bacterial colonies were characterized, counted and calculated as total cfu/g of soil.

3.7. Fluorescence test

Several bacterial strains pathogenic on and associated with onion were tested for possible fluorescence on OEM in comparison with fluorescence on Medium B (KB) of King et al. (1954). Strains tested included strain S22a isolated from New York onion soil in 2011 and tentatively identified as *Pseudomonas putida*; strain A14a isolated from a culled onion bulb removed from storage, tentatively identified as *Ewingella* sp. (the tentative identification for strain A14a was based on matching *gyrB* gene fragment sequences with those available from http://atol.genetics.wisc.edu/). Also included were *E. cloacae* CU6931 isolated from onion (Zaid et al., 2011) and *Pseudomonas glycinea* race 4, CU3395. All strains first were streaked on LB agar then incubated at 28 °C for 24 h. Fresh colonies from these plates were streaked on OEM and KB plates and incubated at 28 °C for 24 h. Fluorescence was observed on a Fotodyne (FOTODYNE Incorporated, Hartland, Wisconsin) transilluminator emitting light at 312 nm. The test was repeated several times using OEM prepared using onions of different varieties from different sources.

3.8. Tests for pathogenicity of bacterial strains in onion leaves, bulbs and sets

Bacteria were tested for their ability to cause infection in onion leaves by piercing leaves with previously sterile toothpicks that had been dipped in freshly prepared bacterial suspensions. The inoculated onion plants were incubated in the laboratory for 5 days, then scored for lesion development. Sterile water was used as a negative control.

Onion bulb and set pathogenicity tests were conducted according to Zaid et al. (2011). Onion slice maceration tests were performed according Kawamoto (1966) with *B. cepacia* strain CU 6878 as a positive control.

4. Results and discussion

4.1. Development of OEM

A broth derived from autoclaving diced pungent yellow onions, adjusted in pH and salt concentration and supplemented with certain inhibitors and agar was found suitable for the selective and rapid growth of onion-pathogenic and onion-associated bacteria in Petri plates. OEM was suitable for initial isolation of bacteria from a variety of onion tissues including seeds, leaves, stalks, bulbs, transplants and from muck-land soil in which onions had grown.

Onion bulbs constitute the only source of nutrients in OEM. They contain 9.34% carbohydrates, which include 4.24% sugars, 1.1% protein, trace minerals and 89.11% water [USDA, National Database for Standard References (Dec, 7. 2011)]. These nutrients were sufficient to support the growth of onion-pathogenic and onion-associated bacteria. On the other hand, onions have anti-bacterial activities against

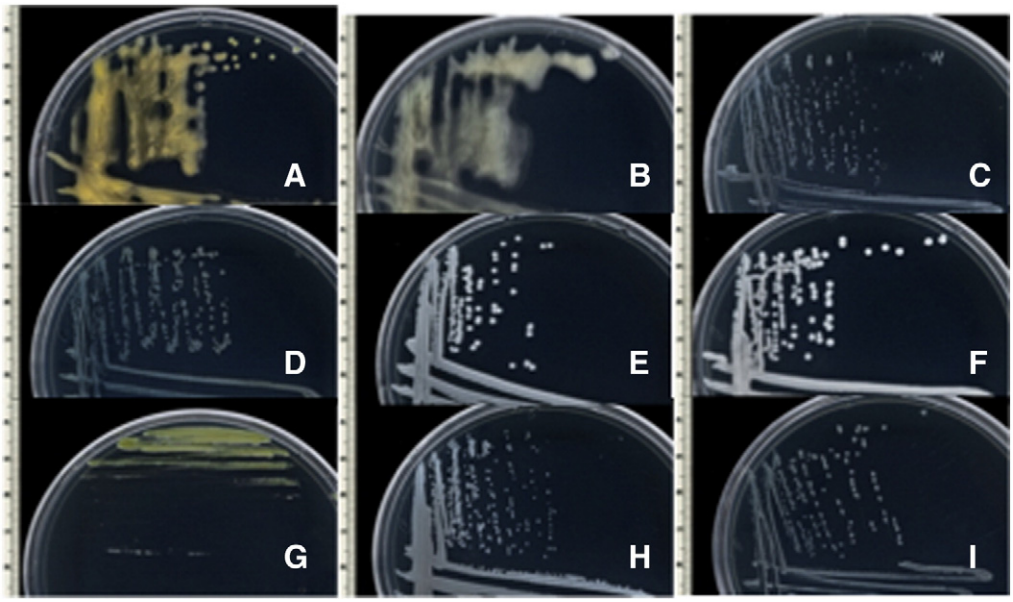

Fig. 1. Characteristics of onion-pathogenic bacteria on OEM. (A) *P. ananatis* CU6829, (B) *P. agglomerans* CU2019, (C) *B. cepacia* CU6878, (D) *B. gladioli* pv. *alliicola* CU3891, (E) *Pseudomonas* sp. CU4236, (F) *E. cloacae* CU6931, (G) *Xanthomonas axonopodis* sub sp. *axonopodis* CU6924, (H) *P. fluorescens* AZ-55, (I) *Pectobacterium carotovorum* sub sp. *carotovorum* CU4148.

many bacterial species including *Vibrio cholera* (Hannan et al., 2010), *Staphylococcus aureus* and *Salmonella enteritidis* (Benkeblia, 2004). The antimicrobial activities of onion bulbs may be due to their content of several organic sulfur compounds (Melvin et al., 2009). OEM also contains crystal violet to inhibit Gram-positive bacteria (Ishimaru and Klos, 1984; Kim and Rhee, 2011) and cycloheximide to inhibit most eukaryotes, such as fungi and yeast (Kawanishi et al., 2011). The concentration of sodium chloride was adjusted to 0.5% (w/v) to reduce mucoid formation and colony size. Potassium phosphate dibasic and potassium phosphate monobasic were used as an acid/base buffer, which affected both colony size and mucoidy.

4.2. Colony morphology on OEM and plating efficiency

4.2.1. Characteristic colony morphology of onion pathogens on OEM

Onion-pathogenic and onion-associated bacteria: *P. ananatis, P. agglomerans, B. cepacia, Burkholderia gladioli* pv. *alliicola, Pseudomonas syringae., E. cloacae, X. axonopodis* subsp. *axonopodis, Pectobacterium carotovorum* subsp. *carotovoum* and *Ewingella sp.* grew well and to a sufficient size after 36 h incubation at 28 °C on OEM for tentative identification based on colony morphology (Fig. 1).

P. ananatis (Fig. 1A) appeared as yellow, opaque, raised mucoid colonies with smooth margins that measured 1.9–3.1 mm in diameter. *P. agglomerans* (Fig. 1B) appeared as pale yellow, raised mucoid colonies with smooth margins that measured 3.8 mm in diameter. *B. cepacia* (Fig. 1C) colonies appeared tiny, cream, opaque, slightly raised, non-mucoid with smooth margins that measured 0.5–0.9 mm in diameter. *B. gladioli* pv. *alliicola* (Fig. 1D) also appeared as tiny, cream, translucent, slightly raised, non-mucoid colonies with irregular margins. *Pseudomonas* sp. (Fig. 1E) appeared as tiny, opaque creamy yellow, slightly raised non-mucoid colonies with irregular margins that measured 0.4–1.2 mm in diameter. *E. cloacae* (Fig. 1F) colonies were shiny, cream-colored, opaque, raised, and non-mucoid with smooth margins measuring 1.9–2.3 mm in diameter. *X. axonopodis* pv. *axonopodis* (Fig. 1G) appeared as yellow-green opaque, raised, moderately mucoid colonies 0.4–0.8 mm in diameter. Colonies of *Pseudomonas fluorescens* (Fig. 1H) appeared yellow-green, opaque, non-mucoid, slightly raised with irregular margins measuring 0.5–1.8 mm in diameter. *P. carotovorum* sub sp. *carotovorum* (Fig. 1I) appeared as tiny cream, slightly raised, non-mucoid opaque colonies with dark centers surrounded by a translucent edge with irregular margins measuring 1.5–2 mm in diameter.

Table 2
Plating efficiency of onion-pathogenic bacteria on OEM medium relative to LB agar.

Bacterial strain	Designation	*Bacterial growth (cfu/5 μl X 10 ± SD) on		Plating efficiency (%) on OEM
		LB	OEM	
P. ananatis	CU6829	110.0 (±43.59)	115.7 (±26.99)	105.18
P. agglomerans	CU2019	301.4 (±30.24)	317.1 (±34.98)	105.21
E. cloacae	CU6931	504.3 (±97.95)	515.7 (±51.92)	102.26
E. cloacae	CU6882	792.9 (±40.30)	797.1 (±28.70)	100.53
B. cepacia	CU6878	108.6 (±38.05)	118.6 (±30.24)	109.21
B. cepacia	CU3368	437.1 (±47.51)	435.7 (±86.0)	99.68
B. cepacia	CU3372	614.0 (±15.74)	640.0 (±23.70)	104.23
B. gladioli pv. *gladioli*	CU3890	132.86 (±24.3)	151.4 (±26.10)	113.95
B. gladioli pv. *alliicola*	CU3891	192.8 (±68.49)	198.33 (±53.45)	102.87
P. fluorescens	AZ-55	811.4 (±137.17)	754.3 (±95.37)	92.96
Pseudomonas sp.	CU4236	152.86 (±41.52)	164.3 (±75.25)	107.48
Xanthomonas axonopodis pv. *allii*	CU6924	141.43 (±33.38)	160.0 (±40.0)	113.13
Pectobacterium carotovorum sub sp. *carotovorum*	CU4148	231.43 (±42.20)	222.86 (±33.52)	96.30
Ewingella sp.	A14a	441.43 (±40.59)	438.57 (±62.03)	99.35

* Mean of seven replicates (± standard deviation).

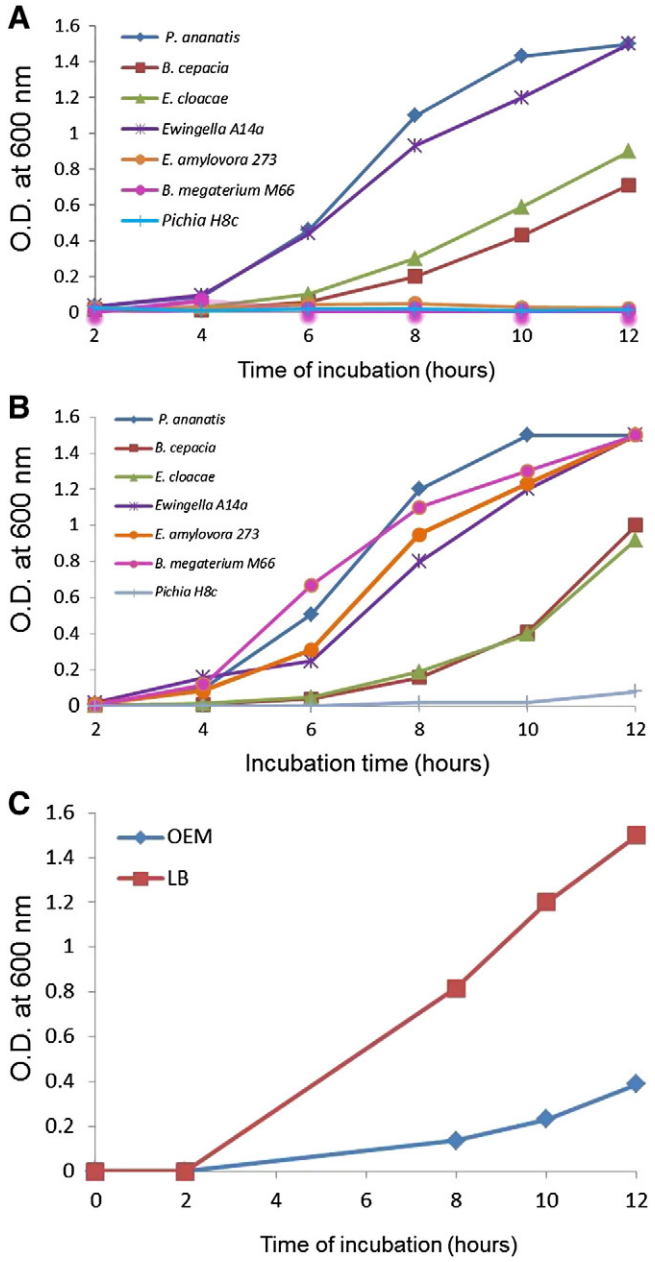

Fig. 2. (A) Growth rate of onion-pathogenic, onion non-pathogenic and onion-associated bacteria in OEB. (B) Growth rate of onion-pathogenic, onion non-pathogenic and onion-associated bacteria in LB broth. (C) Growth rate of *E. coli* DH5α in OEB versus LB broth at its optimum temperature (37 °C).

4.2.2. Plating efficiency of onion-pathogenic and onion-associated bacteria on OEM is similar to plating efficiency on LB agar

Fourteen onion-pathogenic and onion-associated bacteria were selected for assessing plating efficiency of OEM. The plating efficiency ranged from 92.96% for *P. fluorescens* to 113.95% for *B. gladioli* pv. *gladioli* with a mean of 103.46% as compared with LB agar medium (Table 2). Based on the variation in plate counts, the plating efficiency of OEM relative to LB did not differ significantly for the several bacterial strains tested.

4.2.3. Onion-associated bacteria grow more rapidly on OEM than other bacteria

The growth rates of selected onion-associated bacteria, *P. ananatis, E. cloacae*, *Ewingella sp.* and *B. cepacia*, *Bacillus* species and an onion non-pathogen, *Erwinia amylovora*, and *Pichia* species, were determined at 28 °C in OEM broth and LB broth. The onion-associated bacteria *P. ananatis, E. cloacae*, *B. cepacia* and *Ewingella sp.,* had the highest growth rates in OEM broth. The non-pathogen of onion, *E. amylovora*, and onion-associated microorganisms, *Bacillus* species and *Pichia* species H8C, had the lowest growth rates (Fig. 2A). In contrast, the growth rates of the non-pathogens of onion on non-selective LB (Fig. 2B) were higher than the rates of the onion pathogens. *B. cepacia* and *E. cloacae* grew at similar moderate rates in LB broth, while *Pichia* sp. had the lowest growth rate. The slower growth rate of the Gram-positive *Bacillus species* and *Pichia* sp. in OEM broth was expected since it contains crystal violet, an inhibitor of Gram-positive bacteria and cycloheximide, an inhibitor of protein synthesis in eukaryotes.

The growth rate of *E. coli* DH5α at its optimum temperature of growth (37 °C) was less in OEM broth than in LB broth (Fig. 2C). This result confirmed the selectivity of OEM toward bacteria pathogenic to or associated with onions.

4.2.4. Bacteria exhibit fluorescence when cultured on OEM

We tested two fluorescent pseudomonads, *P. putida* strain S22 isolated from NY onion soil and *P. glycinea* race 4 CU3395 isolated from soybean, and two non-fluorescent bacteria, *E. cloacae* CU6931 isolated from onion soil and a putative *Ewingella* sp., A14a, isolated from cull onions, respectively, for fluorescence on OEM compared to the standard medium for fluorescence, King's B (King et al., 1954). The four selected strains were streaked from fresh cultures on plates of OEM and KB media. After incubation at 28 °C for 24 h, fluorescence was observed on a transilluminator. *P. putida* and *P. glycinea* appeared bright on both OEM and KB plates (Fig. 3). The non-fluorescent strains did not fluoresce on either medium.

4.2.5. OEM selectivity

4.2.5.1. Identification of strains recovered on OEM from artificially inoculated and naturally infested onion materials. *P. ananatis, P. agglomerans, B. cepacia,* and *E. cloacae* were recovered from artificially inoculated onion bulbs after incubation for 1 week at 28 °C; onion bulbs inoculated with *E. cloacae* were incubated at 37 °C for 1 week. All recovered strains were streaked on OEM, then transferred to LB agar plates and incubated at 28 °C for 24 h. Identification of the recovered strains was ascertained using biochemical tests (oxidase, indole, nitrate reductase, fluorescence on KB and Gram reaction by testing with 3% (w/v) KOH and by sequencing a portion of the 16 s DNA gene amplified using rp2 and 27 F primers (Weisburg et al.,

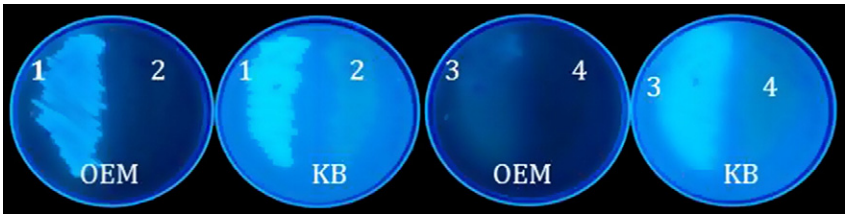

Fig. 3. Fluorescence test for (1) *P. putida* S-22a, (2) *E. cloacae* CU6931, (3) *P. glycinea* race 4 CU3395 and (4) *Ewingella* sp. A14a on OEM, compared to King's Medium B.

1991 and Lane et al., 1991, respectively) as well as a fragment of the *gyrB* gene (J.M. Bonasera et al., unpublished data).

Hundreds of onion-pathogenic and onion-associated bacterial strains were isolated on OEM plates from naturally infested and infected growing onions (bulbs, necks, and leaves), onion planting stock including transplants, seeds and sets. Strains were isolated also from onion soils, harvested onions and onions following storage for 2–8 months. All strains were identified as mentioned above. Thus, we found OEM useful for initial isolation and preliminary characterization of bacteria pathogenic or associated with onions.

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