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Sewage and community shower drains are environmental reservoirs of *Fusarium solani* species complex group 1, a human and plant pathogen

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Summary

In two recent studies, clinical isolates in the *Fusarium solani* species complex (FSSC) were sequenced; one of the most common lineages was FSSC Group 1 (FSSC 1), a phylogenetic species that is synonymous with *F. solani* f. sp. *cucurbitae* race 2, a pathogen of cucurbit fruits. FSSC 1 was also identified in sink and shower drains in two hospitals. The environmental sources of FSSC 1 are important for understanding the epidemiology of both human and plant diseases caused by this organism. FSSC 1 was detected in sewage influent at all six tested urban wastewater treatment plants (WWTPs) in California with a concentration ranging from 75 to 413 colony-forming units (cfu) l^{-1}, a mean of 246 ± 52 cfu l^{-1} and a median of 254 cfu l^{-1}. During the treatment process, the concentration of FSSC 1 in the solid and liquid fractions diminished. FSSC 1 was detected in five and six of 14 community shower drains by culturing and polymerase chain reaction, respectively, whereas FSSC DNA was detected in all drains. FSSC accounted for 17 ± 6% ($n = 14$) of the total fungal DNA in the drains. FSSC 1 was rarely isolated from post-harvest cucurbit fruits and was not found in cucurbit fields in California.

Introduction

Both multilocus DNA sequencing and mating studies indicate that *Fusarium solani* is a species complex comprised of over 45 related, but phylogenetically distinct species. When a sexual cycle is present, *F. solani* are classified as *Nectria haematococca* mating populations (O'Donnell, 2000; Zhang *et al*., 2006; O'Donnell *et al*., 2007) because each species has never been given a formal Latin binomial. Chang and colleagues (2006) concluded that an outbreak of sight-threatening *Fusarium* keratitis associated with contact lens use was caused by multiple lineages primarily in the *F. solani* species complex (FSSC) and *Fusarium oxysporum* species complex. Although a diversity of *Fusarium* spp. caused the keratitis, one-third (13/39) of the isolates from patients in the USA were in a single species that was previously designated as FSSC Group 1 (FSSC 1) (Zhang *et al*., 2006). In a more extensive sequence analysis of 91 isolates associated with the *Fusarium* keratitis outbreak, O'Donnell and colleagues (2007) concluded that FSSC 1 was the most common cause of the corneal infections (29%). In a separate study that also used multilocus DNA sequencing to identify FSSC isolates that cause disease in humans, Zhang and colleagues (2006) determined that 14% of the 278 FSSC isolates from human clinical specimens were FSSC 1; these isolates primarily were from skin ($n = 18$), nails ($n = 6$) and corneas ($n = 5$). Infections caused by FSSC are important, partly because infections in cornea, skin, nails and traumatized tissue occur regardless of immunocompetency. In immunocompromised individuals, *Fusarium* infections that become systemic are often lethal (Nucci and Anaissie, 2002).

In an attempt to determine environmental reservoirs of human pathogenic FSSC, Zhang and colleagues (2006) also sequenced members of the FSSC from a variety of environmental sources including plants and plant debris, animals, soil and hospital environments. Using a collection of FSSC from a cancer hospital (Anaissie *et al*., 2001), Zhang and colleagues (2006) determined that four of five isolates from shower drains and two of nine isolates from sink drains were FSSC 1. Partly because FSSC 1 had been found in sink drains, Chang and colleagues (2006) suggested that splash from sink drains into contact lens cases was a potential source of inoculum for the *Fusarium* keratitis outbreak. In a further analysis of the keratitis cases reported in Chang and colleagues (2006), O'Donnell and colleagues (2007) typed 100 isolates from keratitis patients' 'environments', i.e. their opened bottles of contact lens solution, contact lenses and/or lens cases. FSSC 1 was the most common environmental isolate (32%). Because the remaining isolates were genetically diverse, and *Fusarium* spp. were not isolated from unopened bottles of the contact lens cleaner that was

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Table 1. Primers used in this study.

Specificity	Primer[a]	Sequence (5′–3′)	Binding site	Amplicon size (bp)	Reference	Annealing temperature
Fusarium solani species complex Group 1 (FSSC 1)	Fsc2-EF1 (F)	GTTGGTGACATATCTCCC	TEF	425	Mehl and Epstein (2007b)	55°C
	Fsc2-EF3 (R)	GAGTGAGAGACATGACGG	TEF			
Fusarium solani species complex	AFP346 (R)[b]	GGTATGTTCACAGGGTTGATG	ITS 1	104	Lievens *et al.* (2006)	60°C
Fungi	ITS1-F (F)	CTTGGTCATTTAGAGGAAGTAA	18S rDNA	Variable	Gardes and Bruns (1993)	55°C
	ITS4 (R)	TCCTCCGCTTATTGATATGC	28S rDNA		White *et al.* (1990)	

a. (F), forward; (R), reverse.
b. Used with ITS1-F.
TEF; translation elongation factor 1-α; ITS; internal transcribed spacer.

associated with the outbreak, O'Donnell and colleagues (2007) concluded that patient's bathroom sink drains were a likely source of inoculum.

FSSC 1 is conspecific, i.e. is indistinguishable by multilocus DNA sequence, with *F. solani* f. sp. *cucurbitae* race 2 (Fsc2) (teleomorph, *N. haematococca* mating population V), a plant pathogen that causes fruit rot on cucurbits including pumpkins and squash (Toussoun and Snyder, 1961; O'Donnell, 2000). Previously (Mehl and Epstein, 2007a), we demonstrated that clinical and hospital environmental isolates of FSSC 1 from the Zhang and colleagues (2006) study were as pathogenic on cucurbit fruits as plant isolates of FSSC 1. In addition, clinical and hospital environment isolates were interfertile with plant isolates and therefore members of the same biological species, *N. haematococca* mating population V. Also, clinical, hospital environment and plant isolates were indistinguishable in a variety of phenotypes including the ability to germinate, grow and sporulate at human body temperature. Thus, Fsc2 and FSSC 1 are synonyms. As far as we know, FSSC 1 is unique among the FSSC groups in that it includes isolates that are both human (Chang *et al.*, 2006; Zhang *et al.*, 2006; O'Donnell *et al.*, 2007) and plant pathogens.

Although most cucurbit fruits are potential hosts for FSSC 1 (Toussoun and Snyder, 1961), it is unclear whether infected fruits are an inoculum source for human infections. As a plant pathogen of cucurbit fruits, FSSC 1, i.e. Fsc2, only has been reported in California (CA) and Ohio in the USA (Toussoun and Snyder, 1961), and internationally, in New Zealand (Hawthorne *et al.*, 1992; 1994) and Japan (Suga *et al.*, 2000). In comparison, clinical isolates of FSSC 1 came from people living in 18 different states in the USA and from a total of six countries (Chang *et al.*, 2006; Zhang *et al.*, 2006). It is also unclear whether the FSSC 1 in sink and shower drains from a hospital in the USA (Anaissie *et al.*, 2001; Zhang *et al.*, 2006) and in the contact lenses and storage containers of patients with keratitis (O'Donnell *et al.*, 2007) were a source of inoculum and/or a depository of inoculum that was acquired elsewhere. In the hospital study, only one clinical isolate from a cancer patient (Anaissie *et al.*, 2001) was FSSC 1 (Zhang *et al.*, 2006). However, in the keratitis outbreak, multilocus DNA sequence typing demonstrated that of the 10 matches of patient and environmental isolates, four were FSSC 1 (O'Donnell *et al.*, 2007).

The prevalence of FSSC 1 in either drains, humans or cucurbits is not known. Identification of environmental reservoirs of FSSC 1 is essential for understanding the epidemiology of both the human and plant disease caused by this organism. In this study, we provide the first reports of FSSC 1 (i) in drains in non-hospital settings (some gymnasium and community swimming pool showers) and (ii) routinely in the influent to sewage treatment plants in the Davis, CA area. FSSC 1 was only occasionally observed as a post-harvest disease on cucurbit fruits in the Davis, CA area.

Results

Specificity of primers

The FSSC 1-specific primers (Table 1) amplified 35 clinical, 10 hospital environment, and 7 sewage isolates of FSSC 1 that were previously identified by multilocus sequence (Zhang *et al.*, 2006; Mehl and Epstein, 2007b). The polymerase chain reaction (PCR) products were the predicted size of 425 bp. Cucurbit, sewage and drain isolates collected as part of this study were identified as FSSC 1 with FSSC 1-specific primers.

Quantification of FSSC 1 in sewage wastewater and in the wastewater treatment process

FSSC 1 was present in sewage from 82% ($n = 9/11$) of the campus buildings sampled including lecture halls ($n = 3$), a dining hall ($n = 1$), gymnasiums ($n = 2$), campus housing ($n = 1$) and biological laboratories ($n = 2$) at concentrations ranging from 20 to 388 colony-forming units (cfu) l^{-1} and with a mean of 113 $\pm$ 38 cfu l^{-1}. FSSC 1 was recovered from the sewage effluent on each of 14 sampling times during a 1-year period from one campus gymnasium

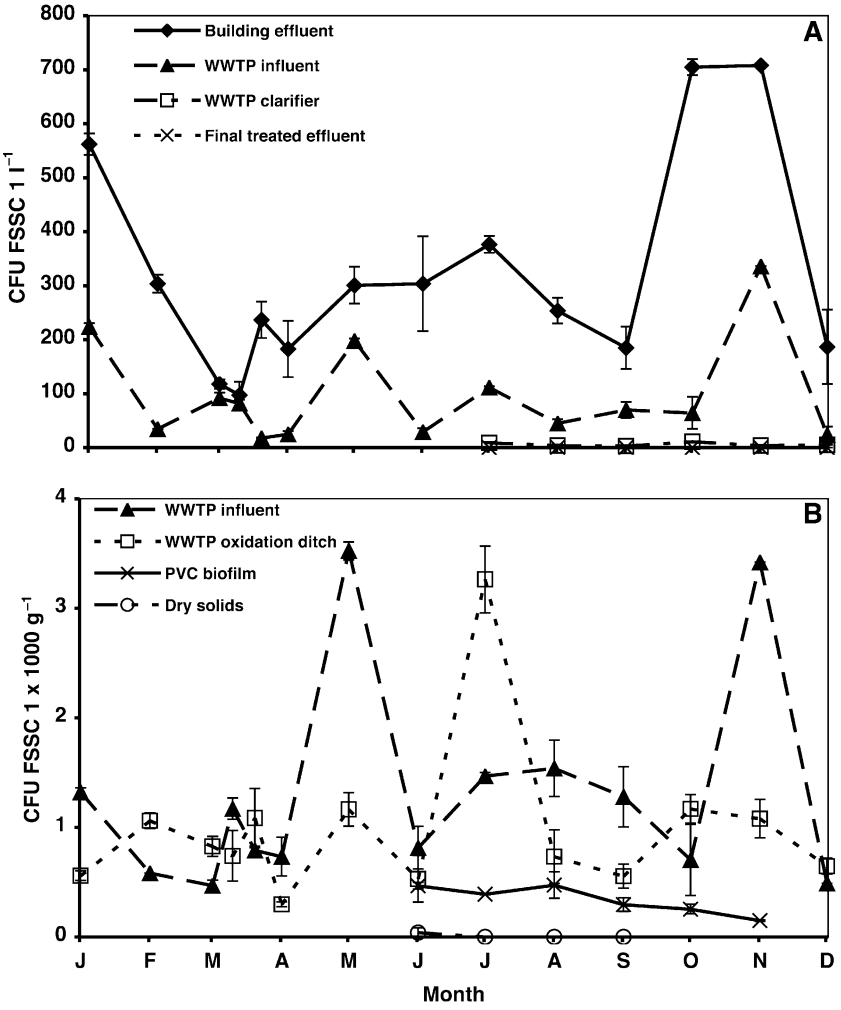

Fig. 1. Quantification of *Fusarium solani* species complex Group 1 (FSSC 1) by liquid volume (A) and dry weight of solids (B) from wastewater collected from a gymnasium and from different locations in the wastewater treatment plant (WWTP) at the University of California at Davis from January through December 2006. Samples were collected on three consecutive days in March and once a month for all other months. CFU, colony-forming units.

(Fig. 1A and B, Table 2). Gymnasium effluent had a mean concentration of 323 ± 53 cfu l^{-1}, and was higher ($P < 0.0001$, paired *t*-test, $n = 14$ sample dates) than the concentration of FSSC 1 (96 ± 25 cfu l^{-1}) entering the University of California (UC) at Davis Wastewater Treatment Plant (WWTP) on every sampling date. However, the quantity of sewage solids present in the gymnasium effluent (178 ± 23 mg of dry solids l^{-1}) was more than twice the amount present in the WWTP influent (76 ± 14 mg l^{-1}). On a dry weight of sewage solids basis, gymnasium effluent had a mean concentration of 2305 ± 541 cfu FSSC 1 g^{-1}, which was significantly greater ($P = 0.023$, paired *t*-test, $n = 14$ sample dates) than the WWTP influent (1307 ± 263 cfu g^{-1}); however, on a dry weight basis, the concentration in the gymnasium effluent was greater on only nine of the 14 sample dates (Fig. 1B).

FSSC 1 was isolated from influent at all ($n = 6$) Sacramento area WWTPs tested with concentrations ranging from 75 to 413 cfu l^{-1} with a mean of 246 ± 52 cfu l^{-1} and a median of 254 cfu l^{-1}. FSSC 1 also was recovered on each of the 14 sampling times during a 1-year period at the entrance to the UC at Davis WWTP (Fig. 1). The concentrations of FSSC 1 per litre (Fig. 1A) and per gram of sewage solids (Fig. 1B) at different stages in the WWTP process are shown in Table 2 so that the effect of the wastewater treatment can be evaluated. On a per litre and per gram of sewage solids basis, there was a significant difference in FSSC 1 concentrations between sample sources ($P < 0.0001$ and $P < 0.0001$ respectively) and sampling dates ($P < 0.0001$ and $P < 0.0001$), and a significant sample source by sample date interaction ($P = 0.0002$ and $P < 0.0001$). Compared with the WWTP influent, there was a significantly lower concentration of FSSC 1 per gram in the WWTP dry solids ($P < 0.0001$, Dunnett's). Two cfu of FSSC 1 were detected in 75 mg of plated dry solids in June; FSSC 1 was not detected on the three later sampling dates (Fig. 1B). The clarifier did not have enough solids to quantify, and solids were absent from the final treated effluent. Scant quantities of FSSC 1 were found in the final stages of wastewater treatment: 11 cfu or fewer were detected per litre in the clarified wastewater, and only 1 cfu of FSSC 1 was detected in 2 l

Table 2. Concentration of *Fusarium solani* species complex Group 1 (FSSC 1) per litre and per gram of solids at different locations in the wastewater treatment plant (WWTP) process at the University of California at Davis in 2006.

	First observational time sequence				Second observational time sequence			
Sampling location[a]	Input months[b]	Sampling period (No. sampling dates)[c]	cfu FSSC 1 g^{-1} dry wt (SE)	cfu FSSC 1 l^{-1} (SE)	Input months	Sampling period (No. sampling dates)[d]	cfu FSSC 1 g^{-1} dry wt (SE)	cfu FSSC 1 l^{-1} (SE)[d]
Gymnasium effluent	Jan–May	Jan–May (7)	1746 (527)	257(59)	Jun–Dec	Jun–Dec (7)	2864(942)	388 (86)
WWTP influent	Jan–May	Jan–May (7)	1227 (400)	96 (32)	Jun–Dec	Jun–Dec (7)	1388 (371)	97 (41)
WWTP oxidation pond	May '05–May	Jan–May (7)	818 (119)	NA	Jun–Dec	Jun–Dec (7)	1139 (366)	NA
WWTP clarifier	–	–	NA	–	Jun–Dec	Jul–Dec (6)	NA	(1)[e]
WWTP final treated effluent	–	–	NA	–	Jun–Dec	Jul–Dec (6)	NA	0.08 (0.08)[e,f]
WWTP dry solids	May '05–May	Jun–Sep (4)[g]	10 (10)[h]	NA	–	–	–	–

a. The gymnasium is the first building on one of the sewage lines to the WWTP. After the raw sewage enters the WWTP, the influent flows into the oxidation pond. In the oxidation pond, the liquid and solids are separated. The liquid flows into a clarifier where additional solids are removed by settling. The wastewater is then UV-treated before the final effluent is released into a creek. Solids from the oxidation pond are moved to drying fields in May, dried during the summer months and then transported to a landfill in the fall.
b. Because oxidation pond solids are moved to the drying fields annually in May, the oxidation pond and the dry solids are a composite of all of the sewage solids from the previous year. All input months were in 2006 except when specifically indicated as from 2005.
c. Samples were collected monthly, except for the clarifier and final treated effluent samples, which were collected when indicated (Fig. 1). On three consecutive days in March 2006, samples also were collected from the gymnasium, WWTP entrance (influent) and WWTP oxidation pond.
d. Because clarifier and final treated effluent were only collected from July through December 2006, the analysis on a per litre basis is limited to six sampling dates during this period.
e. FSSC 1 was significantly lower ($P < 0.0001$, Dunnett's on log-transformed values) in clarified effluent and final treated effluent than in the WWTP influent that contributed to these effluents.
f. FSSC 1 was isolated from a single final treated effluent sample in August 2006.
g. Solids are dried only during the summer months.
h. FSSC 1 was isolated from the solids in the drying bed in June 2006 but not on subsequent sampling dates. FSSC 1 was significantly lower ($P < 0.0001$, Dunnett's on log-transformed values) in dry solids than in the WWTP influent that contributed to these solids.
NA, not applicable; –, samples were not collected; wt, weight.

of the final UV-treated effluent on a single sampling date (Fig. 1A). The clarified wastewater and final treated effluent had significantly lower concentrations of FSSC 1 per litre ($P < 0.0001$ and $P < 0.0001$, respectively, Dunnett's, $n = 6$ sample dates) than the WWTP influent.

To determine if FSSC 1 can form a biofilm on standard plumbing, sections of PVC pipe were submerged in WWTP influent. The concentration of FSSC 1 per gram of biofilm on PVC pipe was 318 ± 35, which was significantly lower ($P = 0.0026$, paired *t*-test, $n = 6$ sample dates) than the concentration per gram of solid in the wastewater in which it was submerged (Fig. 1B). Concentrations of FSSC 1 on PVC pipe submerged for 2–6 months were not higher than those submerged for 1 month (Fig. 1B).

Quantification of FSSC 1 from shower drains

FSSC 1 was quantified by culturing, and FSSC 1, total FSSC and total fungal DNA were quantified by real-time PCR using the taxon-specific primers shown in Table 1. Among the 14 shower drains, FSSC 1 was detected by culturing in 36% ($n = 5$) of the drains sampled (Table 3). When detected by culturing, the concentration of FSSC 1 ranged from 1 to 100 cfu per milligram of dry solids. The arithmetic mean concentration of FSSC 1 in positive samples was 42 ± 18 cfu mg^{-1}.

In each of the shower drain samples, real-time PCR indicated that the amount of FSSC 1 DNA was less than FSSC DNA which was less than the total fungal DNA. FSSC 1 was quantified in 14% ($n = 2/14$) of the shower drain samples by real-time PCR. FSSC 1 comprised 83% and 1% of the total FSSC DNA and 7% and 0.7% of the total fungal DNA in UC Davis Gymnasium 1-F and Gymnasium 2-F respectively.

Quantification of FSSC 1 with real-time PCR was limited by inhibitors in the samples; at the higher levels of taxa, the combination of modifications to the manufacturer's DNA purification protocol and dilution of the template DNA resulted in reasonable estimates of template DNA (Table 3). With FSSC 1, in four of five samples that were positive by culturing, after diluting out inhibitors (a 10- to

Table 3. Quantification of *Fusarium solani* species complex (FSSC) Group 1 (FSSC 1), all FSSC, and all fungi in shower drain biofilms by culturing and real-time PCR.

Location	Shower	FSSC 1, cfu mg^{-1} dry wt (SE)[a,b]	Log (pg DNA mg^{-1} dry wt) (SE)[a,c]		
			FSSC 1	All FSSC	Fungal
Gymnasium 1, UC Davis	M	ND	ND	1.2 (0.2)	1.3 (0.0)
	F	100 (0)	2.6 (0.05)	2.6 (0.1)	3.7 (0.2)
Gymnasium 2, UC Davis	F	ND	1.4 (1.4)	3.4 (0.05)	3.6 (0.2)
Swimming pool 1, Davis	M	ND	ND	2.0 (0.3)	3.1 (0.06)
	F	67 (2)	+/–[d]	1.1 (0.01)	2.7 (0.09)
Swimming pool 2, Davis	M	ND	ND	1.3 (0.4)	3.3 (0.2)
	F	ND	ND	1.0 (0.2)	2.8 (0.06)
Swimming pool 3, Davis	M	14 (3)	+/+[d]	1.1 (0.4)	2.2 (0.2)
	F	28 (4)	+/–	1.6 (0.02)	3.3 (0.08)
Swimming pool 4, Davis	F	0.2 (0.1)	+/–	0.3 (0.09)	1.2 (0.06)
Swimming pool 1, Woodland	M	ND	ND	1.5 (0.2)	2.5 (0.2)
	F	ND	ND	1.2 (0.06)	3.4 (0.2)
Swimming pool 2, Woodland	M	ND	ND	2.4 (0.2)	2.9 (0.3)
	F	ND	ND	2.5 (0.06)	3.1 (0.01)

a. $n = 2$ replicates.
b. Samples were plated onto Komada's medium, incubated at 37°C for approximately 12 h, and then incubated at 25°C. Samples with colony morphology consistent with FSSC 1 were confirmed by PCR.
c. Samples were analysed by real-time PCR as described in *Experimental procedures*.
d. FSSC 1 was detected but was below the limits of accurate quantification for one or both replicates in samples designated with a +/– and +/+ respectively. The limits of quantification and detection were 1 pg and 0.3 pg of DNA per PCR reaction respectively.
M, men's shower; F, women's shower; ND, not detected.

1000-fold dilution), FSSC 1 was below the limits of quantification (LOQ). Because the LOQ for diluted standard DNA was 0.3 pg in a PCR reaction, for samples that were diluted by a factor of 10, 100 and 1000, the LOQ for FSSC 1 was approximately 20, 200 and 2000 pg of DNA mg^{-1} respectively. FSSC 1 was detected and quantified from one shower drain sample (Gymnasium 2-F, UC Davis) in which the fungus was not detected by the plating method.

Isolation of FSSC 1 from cucurbit fruits

Isolates of *Fusarium* were collected from cucurbit fruits in production fields in the San Francisco Bay area, CA, from a produce distributor in San Francisco, and from grocery stores in Davis, CA and the San Francisco Bay Area. In six pumpkin fields in which the pumpkins were presumed to be infected by FSSC 1, 62 pumpkins were infected with *F. solani* f. sp. *cucurbitae* race 1 (teleomorph, *N. haematococca* mating population I), a related but distinct species in the FSSC (FSSC 10) that can also cause fruit rot on cucurbits, and eight were infected with other *Fusarium* spp.; none of the pumpkins had FSSC 1. In a survey for post-harvest *Fusarium* fruit rot (FFR) of cucurbits in a produce distribution facility in San Francisco, CA and in grocery stores, 55 lots of cucurbit fruits had symptoms of *Fusarium*-like fungal rot (Table 4). Isolations were made from lesions, and FSSC 1 was isolated in only four lots from a single grocery store in Davis, CA: once in March 2003 from cucumbers; on two different sampling dates in January 2004 from gourds; and once in January

Table 4. *Fusarium* spp. isolated from post-harvest cucurbit fruits with symptoms consistent with a fungal rot.[a]

Type of cucurbit[b]	No. lots			
	Symptoms consistent with *Fusarium* fruit rot	Infected with *Fusarium*, but not *F. solani* species complex (FSSC)	Infected with FSSC but not FSSC 1[c]	Infected with FSSC 1
Cucumber	11	5	2	1
Gourd	2	0	0	2
Melon	7	2	4	0
Summer squash	17	11	2	0
Winter squash	18	8	3[d]	1

a. Cucurbit fruits were visually inspected monthly from March 2003 through November 2004 at a produce distribution facility in San Francisco, CA and in seven grocery stores in the San Francisco Bay area and in Davis, CA.
b. Summer and winter squash are defined as any squash with a soft and hard rind respectively.
c. FSSC 1, *F. solani* species complex Group 1, a synonym of *F. solani* f. sp. *cucurbitae* race 2.
d. Only one batch was infected with *F. solani* f. sp. *cucurbitae* race 1 (= FSSC 10). FSSC 10 is a more aggressive pathogen than FSSC 1 (Mehl and Epstein, 2007a) and is presumably culled before distribution.

2004 from winter squash. Of the 55 lots of cucurbit fruits with symptoms of fungal rot, 47% ($n = 26$) were infected with *Fusarium* spp. other than FSSC, 20% ($n = 11$) with FSSC other than FSSC 1, and 7% ($n = 4$) were infected with FSSC 1. FSSC 10 was isolated from a single winter squash from the produce distributor in October 2004; however, this reflects the fact that FSSC 10 is primarily a disease in the field and infected fruits are culled before shipment to market. FSSC 1 was not isolated from 54 lots of asymptomatic cucurbit fruits (data not shown).

Discussion

FSSC 1 is a distinct biological and phylogenetic species that is an opportunistic human and plant pathogen (Mehl and Epstein, 2007a); little of its life cycle in the environment is known. Here, we have provided the first report of FSSC 1 in building effluent and in sewage influent; we identified FSSC 1 in six of 12 shower drains in community swimming pools, in raw sewage effluent from nine of 11 buildings, and in sewage influent to six of six urban WWTPs. FSSC 1 was readily isolated in effluent from buildings on the UC Davis campus including three lecture halls in which the only sources of wastewater were from restroom sinks and toilets, and in repeated sampling of effluent from a recently constructed gymnasium in which the primary sources of wastewater were from bathroom sinks, showers and toilets. The higher concentration of gymnasium effluent compared with wastewater influent indicates that the gymnasium was a contributor to the overall load of FSSC 1 in the WWTP. Thus, it is highly unlikely that the inoculum from at least the gymnasium was from discarded food or plant material.

In this study, FSSC 1 was associated with shower drains that have human skin, soil associated with skin, and hair as primary inputs. Whether drains and sewage primarily are a depository of FSSC 1 inoculum from human sources and/or a source of inoculum for new infections remains to be determined. Anaissie and colleagues (2001) concluded that drains and the water supply in a Texas hospital were a source of human infections because FSSC isolated from these sources matched patient isolates based on molecular typing. However, Raad and colleagues (2002) refuted these claims based on a retrospective study of patients in that hospital that had *Fusarium* infections. Our study demonstrates that shower drains used by a presumably relatively healthy population could be a source of FSSC 1. However, it is also conceivable that the inoculum in the drains emanated from soil that was removed during washing. Regardless, splash from shower drains onto skin and nails could serve as an inoculum source. Clinical infections of FSSC 1 are primarily associated with cornea, skin and nails (Chang *et al*., 2006; Zhang *et al*., 2006; O'Donnell *et al*., 2007), tissues that can be infected in immunocompetent individuals without any apparent trauma in the infected tissue (Nucci and Anaissie, 2002; Dignani and Anaissie, 2004; Nir-Paz *et al*., 2004; Chang *et al*., 2006). Ninet and colleagues (2005) suggested that fusarial nail infections could serve as inoculum for disseminated disease in individuals taking immunosuppressive drugs.

Collectively, the FSSC are the most common group of *Fusarium* spp. that cause human fungal infections (Summerbell, 2003; Godoy *et al*., 2004), with isolates that cause infections in humans and other animals concentrated in 'clade 3' of the FSSC (Zhang *et al*., 2006). This is the first study in which FSSC DNA and total fungal DNA in drains were quantified with real-time PCR. Based on real-time PCR, members of the FSSC were present in all 14 drains sampled. FSSC DNA ranged from 0.52% to 69% (arithmetic mean, 17 $\pm$ 6%) of the total fungal DNA in the drains. Previously, Anaissie and colleagues (2001) identified members of the FSSC in drains in a hospital but neither identified nor quantified particular species, or compared the quantity of FSSC with total fungi in the drains. FSSC 1's tolerance of 37°C (Mehl and Epstein, 2007a) would presumably assist in its survival in shower drains.

Concentrations of FSSC 1 in the campus WWTP influent ranged from 18 to 360 cfu l^{-1}, indicating that FSSC 1 is present in untreated sewage. Although we isolated FSSC 1 from newly formed biofilm on PVC pipe submerged in the WWTP influent, the concentrations were lower than in the influent solids, and after the first sampling date, the concentration of FSSC 1 in the biofilm did not increase over time. The limited biofilm formation by FSSC 1 within the wastewater treatment system and the decrease in concentration of the fungus during the treatment process both suggest that FSSC 1 is transient rather than endemic in WWTPs.

FSSC 1 was identified in untreated sewage, which could provide a source of inoculum in some locales both for people and for cucurbit fruits, the plant host. In this study, although post-harvest fruits were collected in California, all four batches of fruit with FSSC 1 were imported from Mexico. However, because only imported fruits from a single grocery store were infected, inoculum may have been acquired post-harvest either during transport or at the store instead of in the field. Regardless, FSSC 1 only was isolated relatively infrequently on post-harvest fruits and was not isolated from cucurbits in the field. Consequently, there is no compelling evidence that either cucurbits or the soil in which they are grown (data not shown) is a common source of FSSC 1 in the environment. In California, some recent diagnoses of FSSC 1 were erroneously based on the morphological identification of FSSC on cucurbits with symptoms that are present on fruits but not obviously on other parts of the plant; identification with taxon-specific primers and translation elongation factor

1-α (TEF) sequence analysis (GenBank Accession No. DQ913762–DQ913767) indicated that misidentified isolates were a related but distinct and more virulent species that can also infect the crown and roots of cucurbits, FSSC 10 (*F. solani* f. sp. *cucurbitae* race 1) (Mehl and Epstein, 2007b). Although further work is required to fully elucidate the life cycle and environmental reservoirs of FSSC 1, overall, our data do not support the hypothesis that infected cucurbits are a major source of inoculum for human infections. In contrast, our study supports the hypothesis that exposure to raw sewage and splash from shower drains could provide a source of FSSC for skin and nail infections in humans. Similarly, although we did not examine sink drains, our data indirectly support the conclusion that splash from sink drains into contact lens cases could provide a source of FSSC for corneal infections, as suggested by Chang and colleagues (2006) and O'Donnell and colleagues (2007).

Experimental procedures

PCR primer specificity

Primers Fsc2-EF1/Fsc2-EF3 (Table 1) from the TEF region were designed previously to specifically amplify plant isolates of *F. solani* f. sp. *cucurbitae* race 2 (Fsc2) (Mehl and Epstein, 2007b). Although Fsc2 and FSSC 1 are synonyms and have identical TEF sequences, to verify that the primers were FSSC 1-specific and could amplify DNA from clinical and environmental isolates from various geographic origins, the primers were tested on a collection of human clinical ($n = 35$) and hospital environment ($n = 10$) isolates of FSSC 1 obtained from K. O'Donnell, National Center for Agricultural Utilization Research, USDA-ARS, Peoria, IL (Zhang *et al.*, 2006). The primers were also tested on FSSC 1 isolates from sewage ($n = 7$) that were collected previously during a preliminary survey for FSSC 1 in the environment (Mehl and Epstein, 2007a,b). DNA extractions from fungal cultures and PCR were performed as described previously (Mehl and Epstein, 2007b).

Isolation and quantification of FSSC 1 from shower drains

Community shower drains in public pools and gymnasiums were selected because they are used by multiple individuals and have sufficient biofilm for sampling. Fourteen biofilm samples (six and eight from showers for males and females respectively) were collected from eight facilities in Davis and Woodland, CA. Samples were suspended in 0.01% Tween 20 for a final volume of 7.5 µl per milligram wet weight. Volumes of 0.25 ml were spread on Komada's medium (Komada, 1975). Five plates each of the initial suspension and 0.5, 0.1 and 0.01 dilutions of the initial suspension were plated for a total of 20 plates per sample. After plating on Komada's medium, samples were incubated at 37°C overnight because this temperature kills some fungal contaminants but is tolerated by FSSC 1. Plates were then incubated at 25°C under lights for 4 days. Putative FSSC 1 colonies were subcultured onto Komada's medium, and identification of colonies with morphology consistent with FSSC 1 were confirmed with taxon-specific primers. Biofilm samples were lyophilized to determine the dry weight of the sample.

A real-time PCR assay was developed to quantify FSSC 1, total FSSC and total fungal DNA from drain samples. The taxon-specific primers are described in Table 1. For standards, DNA was extracted from pure cultures of FSSC 1 using the DNeasy Tissue Kit (Qiagen) and quantified with a spectrophotometer. Dilutions of the DNA from 10^2 pg to 10^{-1} pg in a one-half log series were used as standards for the real-time PCR. DNA was extracted from approximately 40 mg of lyophilized biofilm samples using a QIAamp DNA Stool Mini Kit (Qiagen). Samples were placed in a 2 ml microfuge tube with 1 g of 0.5 mm glass beads and homogenized with a Mini Beadbeater (BioSpec Products, Bartlesville, OK) for 1 min. The manufacturer's protocol was then followed except the washing step was modified to improve removal of PCR inhibitors: Buffer AW1 was added to the column and incubated at room temperature for 2 min before centrifugation; this step was performed a total of three times. The same procedure was repeated with Buffer AW2. The DNA was then eluted with 200 µl of elution buffer.

Real-time PCR amplifications were performed using SYBR Green I with a 7500 Real-time PCR System (Applied Biosystems). Each reaction contained 10 µl of DNA extract, 25 µl of SYBR Green PCR Master Mix (Applied Biosystems), 1 µl each of forward and reverse primer (10 µM), 0.5 µl of BSA (10 mg ml^{-1}, New England BioLabs, Beverly, MA), and 12.5 µl sterile distilled water. Thermal cycling conditions were 50°C for 2 min and 95°C for 10 min followed by 40 cycles of 95°C for 33 s, the annealing temperatures indicated in Table 1 for 33 s and 72°C for 1 min. At the end of the PCR run, the thermal melt temperature of each sample was determined. Because the undiluted DNA extract still contained PCR inhibitors, a dilution series of 10^{-1} to a maximum of 10^{-3} was used for quantification. The absence of PCR inhibitors in quantified samples was demonstrated by obtaining the same estimate of template DNA in two successive dilutions and by quantifying DNA in samples with and without the addition of a known quantity of FSSC 1 DNA. Two independent DNA extractions per samples and at least two PCR reactions per DNA extract were used to estimate picogram of target DNA.

Quantification of FSSC 1 from sewage and wastewater treatment plants

Wastewater effluent samples were collected in February 2005 from 11 buildings on the UC Davis campus including two gymnasiums, two biological laboratories, three lecture halls, two dining halls and two student housing complexes. Because all effluent samples were collected at the terminus of sewer lines, each sewage sample was from the single indicated building. Wastewater samples also were collected from the influent into the UC at Davis between January 2005 and December 2006, the city WWTP in Davis, CA in January 2005 and four other urban WWTPs in Northern California in April 2006: Grass Valley, Auburn, Roseville and West Sacramento. Fungi in wastewater were concentrated by

filtration onto 47-mm-diameter cellulose nitrate membrane filters with 1.0 μm pores (Whatman, Florham Park, NJ), with volumes of 10–25 ml per membrane. After filters were placed upside-down on Komada's medium and incubated at room temperature under lights for 3–4 days, identification of colonies consistent with FSSC 1 on Komada's medium was confirmed with taxon-specific primers.

Monthly during 2006, FSSC 1 was quantified at different points in the UC Davis wastewater treatment system, starting with sewage effluent from a campus gymnasium that was built in 2004 at the terminus of the sewage line. Sources of sewage from this building were showers, restroom sinks and toilets. WWTP influent, which includes all of the raw sewage from campus, was collected at the entrance to the UC WWTP. At the WWTP, influent flows into the oxidation ponds where the sewage solids settle and undergo microbiological treatment, while the wastewater flows to a clarifier. In the clarifier, the remaining solids are filtered out and UV-treated before the final effluent is released from the treatment plant. Because oxidation pond solids are transported to a drying bed in May and allowed to desiccate before transport to a landfill at the end of the summer, dried solids were collected from June through September 2006. Building effluent, WWTP influent and oxidation pond samples were collected once monthly from January 2006 through December 2006 with the exception of March during which samples were collected on three consecutive days. Liquid from the clarifier and the final WWTP effluent were collected from July through December 2006.

Building effluent, WWTP influent and oxidation pond samples were centrifuged to concentrate the suspended sewage solids. A total of 1 l of building effluent and 4 l of WWTP influent per sample was centrifuged at 10 000 *g* for 20 min. The resulting pellets were re-suspended in approximately 100 ml of the liquid and centrifuged at 20 000 *g* for 20 min. In addition, 100 ml of oxidation pond solids were similarly centrifuged at 20 000 *g*. The liquid was decanted, and the concentrated solids were weighed. After solids were suspended in 7.5 ml of 0.01% Tween 20 per gram wet weight, 0.25 ml were spread evenly onto each of 10 plates of Komada's medium. A 0.5 dilution of the original suspension also was plated in a similar manner. Subsamples of the solids were dried at 50°C for 5 days to determine the dry weight of the samples. In the text, per milligram always refers to dry weight.

After dry solids collected from the WWTP were homogenized with a mortar and pestle, 1 g was suspended in 50 ml of 0.01% Tween 20. The suspension and a 0.5 dilution of the suspension were plated as described above. Because the clarified wastewater and the final treated effluent had too few solids for quantification, liquid was filtered through a 1.0 μm cellulose nitrate membrane filter (Whatman, Florham Park, NJ). Per sample, a total of 1 l in 100 ml aliquots and 2 l in 200 ml aliquots was filtered for the clarified wastewater and final effluent respectively. Filters were placed upside-down on Komada's medium. Plates were incubated overnight at 37°C and then at 25°C as described above for the shower drain biofilm samples. Putative FSSC 1 colonies were subcultured onto Komada's medium, examined for colony morphology consistent with FSSC 1 and confirmed with taxon-specific primers.

Because pipes within the wastewater treatment system were not available for sampling, in order to study biofilm formation within the system, sections of 5 cm diameter by 10 cm length PVC pipe were submerged in the influent at the WWTP. Pipes were submerged in May 2006 (time = 0) and five pipes were removed each month from June through November 2006 so that biofilm development after 1 through 6 months of contact with wastewater was observed. Biofilm was scraped off of the pipe, and cfu of FSSC 1 were quantified as described above.

Isolation of FSSC 1 from cucurbits

Cucurbit fruits in both the field and post-harvest were surveyed. University of California cooperative extension farm advisors in the cucurbit-growing regions of California indicated that disease that was presumably caused by FSSC 1 had been observed recently only on pumpkins in the Pescadero/Half Moon Bay region. *Fusarium* fruit rot symptoms occurred during the autumn months, and in October of 2004 through 2006, six pumpkin fields had symptoms of FFR presumably caused by FSSC 1. At least 100 pumpkins per field were observed, and isolations were made from 70 pumpkins with symptoms of FFR. Tissue from the margin of FFR lesions was plated on *Fusarium* selective medium (FSM) (Mehl and Epstein, 2007b). Cucurbit fruit samples with possible FFR were also collected from an organic produce distributor in San Francisco, CA and from seven grocery stores in Davis, CA and the San Francisco Bay area. Cucurbits representative of common cultivars were collected from the following categories: cucumbers (*Cucumis sativus*), gourds (*Cucurbita pepo*), melons (*Cucumis melo*), summer/soft rind squash (*C. pepo*) and winter/hard rind squash (*C. pepo*, *C. maxima*, *C. moschata*). Samples were collected monthly from March 2003 through November 2004. For each variety of cucurbit fruit available on a given sampling date, fruit lots were inspected for the presence of lesions suggestive of a fungal infection. Depending on availability, a batch of fruit consisted of 10 or more individual cucurbit fruits. Symptomatic fruits from each of the cultivar categories were not present on all sampling dates, but isolations were made from all fruit lots that had symptoms of FFR. A total of 55 symptomatic and 50 asymptomatic cucurbit fruits or batches of fruits were selected from 105 cucurbit fruit lots. Tissue samples were plated on FSM and FSSC and other *Fusarium* spp. were selected based on colony morphology for further characterization. For both field and post-harvest isolations, colonies that were putatively identified as FSSC based on colony morphology were screened with taxon-specific primers for FSSC 1 as described previously (Mehl and Epstein, 2007b).

Data analysis

For samples collected in the wastewater treatment system, two replicates of five plates each were used to estimate cfu. Colony-forming units per litre were calculated based on the dry weight of solids obtained from the total volume of sample centrifuged. Except when a paired *t*-test is indicated, an analysis of variance was used to compare concentrations of FSSC 1 at different locations in the treatment process. For ANOVA, data were log(cfu + 1) transformed to meet the assumptions of homogeneity of variance and normality, and means were

compared using a Dunnett's with the WWTP influent as the standard with which other means were compared.

DNA was extracted from two subsamples from each shower drain with a subsample from each drain in each of two batches; there was no significant difference between extraction batches ($P = 0.45$). Each sample was analysed in two separate real-time PCR runs. Because there was no significant difference in DNA quantification between real-time PCR runs ($P = 0.32$), results from runs were averaged. Data were log transformed to meet the assumptions of homogeneity of variance.

All statistical analyses were performed using JMP 5.1 (SAS Institute). In the text, arithmetic means of the untransformed data ± standard error are shown.

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